THE

BIOCHEMICAL JOURNAL

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Identification of the Uronic Acid from Oestriol 'Monoglucuronide'

By J. K. GRANT and G. F. MARRIAN
Department of Biochemistry, University of Edinburgh

(*Received 2 February* 1950)

In 1936 Cohen & Marrian isolated from human pregnancy urine an amorphous water-soluble substance containing about 50% of combined oestriol. From its elementary composition and properties this substance appeared to consist largely of an oestriol monoglucuronide. Subsequently (Cohen, Marrian & Odell, 1936) this substance yielded a crystalline sodium salt, which, on the basis of its elementary composition and a strongly positive Tollens naphthoresorcinol reaction, appeared to be nearly pure sodium oestriol glucuronidate. In the course of this work it was shown that the uronic acid moiety is attached to the oestriol by a glycosidic linkage involving the potential aldehyde group of the former, and the C-16 or C-17 hydroxyl group of the latter. No clear-cut evidence was obtained, however, that the uronic acid is indeed glucuronic acid. In view of the lack of specificity of the Tollens reaction for glucuronic acid (Mandel & Neuberg, 1908; Dische, 1946) it was felt that a further investigation of the identity of the uronic acid component should be undertaken.

The identification of uronic acids is difficult, and the choice of method was restricted by the small quantity of purified oestriol 'monoglucuronide' which could be prepared. Unequivocal methods such as the isolation of D-glucurone as used by Pryde & Williams (1933) in the case of borneol glucuronide, or the preparation of the *p*-toluidine complex of ammonium glucuronidate (Smith & Williams, 1949) are not recommended for work with small quantities of glucuronide (Williams, 1949). The method of Lohmar, Dimler, Moore & Link (1942) involving oxidation of a uronic acid to a dicarboxylic acid, with subsequent identification of the latter as the dibenziminazole derivative, is open to the criticism that it will not distinguish between D-glucuronic and L-guluronic acids as both of these acids give D-glucosaccharic acid on oxidation. The method has however been employed by Levvy (1948) working with small quantities of menthyl glucuronide, and although Bernhauer & Irrgang (1935) believe that they have demonstrated the production of L-guluronic acid by the action of certain bacteria on glucose, this acid has not so far been found to be produced by animal organisms. It therefore appeared that this method would provide a means of identifying the uronic acid moiety of oestriol 'monoglucuronide' with reasonable certainty.

The hydrolysis necessary for the liberation of the uronic acid from combination with oestriol presented certain difficulties. The work of Stevenson & Marrian (1947) on the acid hydrolysis of conjugated oestrogens in human pregnancy urine suggested that the complete hydrolysis of oestriol 'monoglucuronide' may require as long as 30–40 min. boiling with addition of 15% by volume of concentrated hydrochloric acid. It was felt that such harsh treatment might result in extensive destruction of the uronic acid liberated. Accordingly milder means of hydrolysis were sought.

Odell, Skill & Marrian (1937) and Fishman (1939) showed that preparations of β-glucuronidase obtained from animal tissues will liberate oestriol from sodium oestriol 'monoglucuronidate', and Levvy (1948) has shown that this enzyme will liberate glucuronic acid from menthyl glucuronide. These facts suggested that hydrolysis by the action of glucuronidase might be suitable for the present purpose.

Sodium oestriol 'monoglucuronidate', purified as thoroughly as existing methods permit, was incubated in two portions with a purified ox-spleen β-glucuronidase preparation, and gave uronic acid fractions in yields of about 62 and 79% of theory. After oxidation with bromine, and treatment with *o*-phenylenediamine, a product was obtained which was satisfactorily identified as the dibenziminazole of D-glucosaccharic acid. It is clear therefore that the uronic acid obtained by the enzymic hydrolysis of oestriol 'monoglucuronide' was either D-glucuronic acid or L-guluronic acid. In view of the improbability that the latter is produced in animal organisms, it seems reasonable to assume that the uronic acid is indeed D-glucuronic acid. No evidence has been obtained regarding the ring structure or anomeric form of the uronic acid. Glucuronic acid is at present known to occur naturally only as the β-form.

A preliminary communication on this work has already been made (Grant & Marrian, 1948).

EXPERIMENTAL

All melting points referred to in this section are corrected.

Preparation of sodium oestriol 'monoglucuronidate'

Na oestriol 'monoglucuronidate' was prepared from human pregnancy urine by the method of Cohen *et al.* (1936) with various modifications. The troublesome emulsions which

frequently form when urine is shaken with butanol, were avoided by adopting a modification of the procedure of Robinson & Warren (1948). These workers found that emulsions did not form with $CHCl_3$ if it was injected in the form of a fine spray under the surface of the urine which was being extracted. In the present work the urine was very conveniently extracted by spraying into columns of *n*-butanol. The *n*-butanol, purified by refluxing with NaOH and distillation, was contained in glass tubes 150 cm. long and 4 cm. diameter. The urine flowed into the columns, under gravity, through a number of fine capillaries opening under the surface of the butanol. No emulsions formed, and the pool of partially extracted urine collected at the bottom of the column was siphoned off continuously, and allowed to flow twice through a fresh column of butanol. In order to deal with larger batches of urine the columns were set up in batteries of three. After the extraction, butanol from the columns was pooled for further treatment.

The urine was collected in batches of 5–10 l., acidified to pH 3·5 with HCl, and extracted with *n*-butanol as described. The pooled butanol extracts were brought to pH 7 with 10% (w/v) NaOH in butanol, concentrated under reduced pressure to $\frac{1}{5}$ volume, and extracted three times with $\frac{1}{10}$ volumes of 0·33 N-NaOH. The combined NaOH extracts were stored in the refrigerator. When similar alkaline extracts from a total of 50 l. of urine had been collected they were combined, acidified to pH 3·5, and extracted three times with $\frac{1}{3}$ volumes of butanol. The combined butanol extracts were washed twice with $\frac{1}{10}$ volumes of water, made alkaline with 10% (w/v) Na_2CO_3 and evaporated to dryness under reduced pressure. The brown residue was dissolved in 500 ml. water, and the solution, after acidification to pH 3·5, was washed six times with 100 ml. volumes of benzene. Crude Na oestriol 'monoglucuronidate' was obtained from the benzene-washed aqueous solution by the quinoline extraction and subsequent procedures described by Cohen *et al.* (1936). The average yield of the crude salt obtained at this stage was 10 mg./l. of urine.

The crude product was purified in the following way: repeated leaching of the solid with hot moist butanol dissolved the Na oestriol 'monoglucuronidate' leaving a small amount of undissolved dark-coloured tar. The combined butanol leachings were extracted three times with $\frac{1}{10}$ volumes of 0·33 N-NaOH; the combined alkaline extracts were acidified to pH 3·5 with HCl, and extracted three times with $\frac{1}{3}$ volumes of butanol. The butanol extract was washed twice with $\frac{1}{10}$ volumes of water, and taken to dryness under reduced pressure. The residue was dissolved in about 500 ml. methanol, made just alkaline with a methanolic solution of NaOH, boiled with a small amount of Norit charcoal, and filtered while hot. The filtrate was concentrated to about $\frac{1}{5}$ volume. During the concentration Na oestriol 'monoglucuronidate' separated as a nearly white crystalline solid. After chilling, the solid was filtered, washed with a little cold methanol, and dried *in vacuo*.

Preparation and characterization of oestriol 'monoglucuronidic' acid

Satisfactory C and H analyses were not obtained with material purified as described above; it was therefore converted to oestriol 'monoglucuronidic' acid as follows. The Na salt was dissolved in a minimum of warm moist butanol. The solution was adjusted to pH 1 with HCl, washed with $\frac{1}{10}$ volumes water until the washings were free from chloride, and taken to dryness under reduced pressure. The residue was dissolved in a minimum of boiling ethanol and precipitated from solution with 10 volumes of cold dry ether. The white amorphous solid oestriol 'monoglucuronidic' acid was filtered off and dried *in vacuo*. The product was successfully freed from solvents by repeatedly wetting with warm water, drying over $CaCl_2$ and subsequently over P_2O_5 *in vacuo* at 80°.

The sample of the acid which was reconverted to the Na salt for enzymic hydrolysis had m.p. 224–226°(decomp.); $[\alpha]_D^{17°} - 7{\cdot}5° \pm 0{\cdot}8°$ in ethanol (*c*, 1·061). (Found: C, 62·2; H, 6·9. Calc. for oestriol 'monoglucuronidic' acid $C_{24}H_{32}O_9$: C, 62·0; H, 7·0%.)

Preparation and characterization of sodium oestriol 'monoglucuronidate' for enzymic hydrolysis

The Na salt used for the enzymic hydrolysis was prepared from the pure oestriol 'monoglucuronidic' acid by dissolving the acid in anhydrous methanol, adding an equivalent of NaOH in anhydrous methanol, and evaporating the solution to small volume under an air stream. The white solid which separated was filtered off and washed with cold dry methanol. A sample after drying *in vacuo* at 137° over P_2O_5 for 8 hr. melted at 245–248·5° (decomp.). (Found: C, 59·6; H, 6·5; Na, 4·6. Calc. for Na oestriol 'monoglucuronidate' $C_{24}H_{31}O_9Na$: C, 59·3; H, 6·4; Na, 4·7%.) The dry salt was found to be very hygroscopic. After drying to constant weight over P_2O_5 *in vacuo* at 137°, a sample picked up 17·1% moisture on equilibration with the laboratory atmosphere.

In the previous work (Cohen *et al.* 1936) preparations of the Na salt containing 0·5 molecule of methanol of crystallization and 1·5 molecules of water of crystallization and melting respectively at 305–306° (uncorr.) and 256–257° (uncorr.) were described, but the solvent-free salt was not obtained. However, in a private communication Dr S. L. Cohen has informed the authors that he also has now prepared the solvent-free Na salt.

Preparation of β-glucuronidase

The enzyme was prepared from ox spleen, and its activity determined by the procedures described by Graham (1946). On account of great losses of activity in the final stages, preparations were not purified beyond Graham's stage E.

The hydrolysis of sodium oestriol 'monoglucuronidate' and isolation of the uronic acid as the dibenziminazole derivative of D-glucosaccharic acid

Na oestriol 'monoglucuronidate' (350 mg.) was incubated at 37° in 250 ml. 0·25 M-acetate buffer, pH 4·6, containing 1000 Graham units of β-glucuronidase. Toluene (1 ml.) was added to inhibit growth of bacteria. A further 500 units of enzyme in 45 ml. solution were added after 24 hr. At the end of 3 days protein was precipitated with 4 vols. of dry acetone, and filtered off. The filtrate was evaporated to a volume of 100 ml. It was then acidified with H_2SO_4 and extracted 3 times with 30 ml. ether to remove oestriol. From its reducing power, the ether-extracted aqueous phase appeared to contain 87 mg. uronic acid, corresponding to 62% hydrolysis of the oestriol 'glucuronide'. This uronic acid fraction was oxidized, and the dibenziminazole derivative of the product was prepared in a yield of 47 mg. by the methods described by Lohmar *et al.* (1942) as modified by Levvy (1948).

The hydrolysis was repeated with a further 340 mg. Na oestriol 'glucuronidate'. In this case 79% hydrolysis was indicated and 55 mg. dibenziminazole derivative finally obtained. This was combined with the product of the first experiment before purification.

The dibenziminazole isolated had m.p. 242–243° (decomp.). The mixed m.p. with an authentic sample of the dibenziminazole derivative of D-glucosaccharic acid (m.p. 241–243°, decomp.) was 242–243° (decomp.). The dipicrate and dihydrochloride were prepared as described by Levvy (1948). The dipicrate melted with decomposition at 210° after change of form at 142°; an authentic sample melted at 211° (decomp.), after change of form at 142°, and showed no depression in m.p. after mixture with the material isolated. The dihydrochloride melted at 265–266° and had $[\alpha]_D^{18°}$ +49·8° ± 0·6° in water (*c*, 2·057). On admixture with an authentic sample (m.p. 266–267°; $[\alpha]_D^{18°}$ +49·3° ± 0·6° in water (*c*, 2·042)) the m.p. was unchanged. Levvy (1948) found $[\alpha]_D^{18°}$ +52·3° in water (*c*, 2·024) with his preparation of the dihydrochloride, which he showed to be the tetrahydrate. In order to avoid difficulty with varying degrees of hydration in this work, both the dihydrochloride isolated from the urinary uronic acid and the authentic dihydrochloride were recrystallized from water, and dried over $CaCl_2$, under identical conditions.

SUMMARY

1. A modified method for the isolation and purification of oestriol monoglucuronidic acid and its sodium salt from human pregnancy urine is described.

2. The sodium salt has been hydrolysed by ox-spleen β-glucuronidase. The uronic acid so liberated has been oxidized to D-glucosaccharic acid which has been identified as its dibenziminazole derivative. It is therefore probable that the uronic acid moiety is D-glucuronic acid.

The authors are indebted to the Medical Research Council for a grant from which the expenses of this work were largely defrayed, to Prof. R. J. Kellar for arranging for the collection of pregnancy urine, to Dr J. W. Minnis who carried out the micro-analyses and to Mr D. Brown for skilled technical assistance. Dr G. A. Levvy kindly supplied samples of the hydrochloride and picrate of the D-glucosaccharic acid dibenziminazole, and offered much useful criticism and advice regarding the isolation and characterization of the derivatives from the products of enzymic hydrolysis. The authors are also grateful to Dr S. L. Cohen (University of Minnesota) for allowing them to see the manuscript of his paper before publication.

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Studies on Rhodopsin

1. METHODS OF EXTRACTION AND THE ABSORPTION SPECTRUM

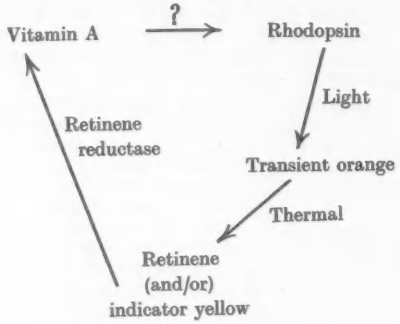
By F. D. COLLINS and R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 27 *October* 1949)

The history of visual pigments has been reviewed on numerous occasions: Granit (1947) has given a comprehensive and invaluable summary. As the result of the work of R. J. Lythgoe and his co-workers and of G. Wald, our knowledge of rhodopsin has been greatly enlarged. This knowledge can be conveniently summarized in the cycle shown on p. 4, based on that first proposed by Wald (1938).

It has been shown that rhodopsin is responsible for scotopic or twilight vision although not for photopic vision. Retinene has been shown in this laboratory (Morton & Goodwin, 1944) to be vitamin A aldehyde, whilst indicator yellow is now thought to be a compound between retinene and a suitably bound amino group (Ball, Collins, Dalvi & Morton, 1949) of the protein moiety.

However, although so much is now known, it would be hazardous to say that rhodopsin has ever been obtained in a state of purity. It is therefore the purpose of the present paper to consider methods of extraction, the nature of the impurities and the degree of purity actually obtained.



All successful methods for the extraction of rhodopsin involve the use of detergents such as bile salts (Kühne, 1878) or digitonin (Tansley, 1931). In the present work aqueous digitonin has been used to extract the rhodopsin from the retina. Many workers have recommended the use of alum in order to harden the retinas before extraction (Kühne, 1878; Chase & Haig, 1938, etc.). Saito (1938), Krause & Sidwell (1938) and Lythgoe (1937) developed different methods for separating the rod outer segments from the main bulk of the retinal tissue.

In the course of this study several of the above methods have been tried either singly or in combination, and for comparative purposes a working criterion of purity ($=P$) has been arbitrarily defined as the ratio of the extinction at $\lambda_{min.}$, generally near to 400 mμ., to that at $\lambda_{max.}$ (cf. Wald, 1938) near 500 mμ. A large number of preparations of rhodopsin has been obtained and the absorption spectrum measured in each.

Some values of P based on published curves or numerical data for rhodopsin solutions obtained by previous workers, are shown in Table 1.

Table 1. *Values for* $P_{\lambda\ min.}$ ($E_{min.}/E_{max.}$) *recorded in the absorption curves of rhodopsin and porphyropsin*

Species from which retinas were obtained	$P_{\lambda\ min.}$	Observer
Frog	0·30–0·39	Lythgoe (1937)
Ox	0·31	Krause & Sidwell (1938)
Bullfrog	0·32, 0·54–0·68	Wald (1938)
Rabbit	0·54	Wald (1938)
Various fresh-water fishes	0·50–0·77	Wald (1939)
Bullfrog	0·40–0·57	Chase & Haig (1938)

Wald (1949) gives $P_{\lambda\ min.}$ 0·26 without stating the species.

EXPERIMENTAL

Solutions

Potash alum. $K_2SO_4.Al_2(SO_4)_3.24H_2O$, 4% (w/v) in water.

Digitonin solution. 2% (w/v) in water unless otherwise stated; prepared by heating the mixture of digitonin and water to boiling until solution occurs and then quickly cooling.

Sucrose. 45% (w/v) in water.

Buffer solutions. pH 9·3, $Na_2B_4O_7.10H_2O$, 19 g./l.; pH 7, 16·4 ml. of 0·2 M-Na_2HPO_4 and 3·53 ml. of 0·1 M-citric acid.

Animals

In the course of this work the following animals were employed as sources of rhodopsin: frogs (*Rana temporaria*), rats (from a small animal colony), sheep and cattle (from the local abattoir), pike (*Esox lucius*), char (*Salvelinus willoughbii*), perch (*Perca fluviatilis*) and haddock (*Gadus aeglefinus*).

Apart from sheep and cattle, the animals were dark adapted for at least 3 hr. and often overnight. In the case of sheep and cattle the eyes were obtained soon after slaughter and kept in the dark until dissected.

The dissection and all subsequent operations were carried out in red light. The rats were dissected by the method of Tansley (1931) and the frogs as described by Lythgoe (1937). The eyes of the other animals had an incision made close to the ora terminalis, the junction of the retina and the conjunctiva. The incision was then continued with a pair of scissors all round the ora terminalis, thus enabling the cornea, lens and aqueous humour to be removed. The vitreous humour was next removed, although sometimes the retina adhered to it. In any case, the retina could then be obtained either from the choroid or from the vitreous humour. In some cases the retinas were hardened *in situ* with alum after the removal of the lens and vitreous humour. This was done with the haddock, pike and char.

Extraction

Four methods were used:

'*No alum*' *method.* In this case the fresh retinas were extracted with 1 or 2 ml. of 2% digitonin, generally for 1 hr. After extraction with digitonin the mixture was centrifuged strongly, the supernatant liquid containing the rhodopsin.

'*Alum*' *method.* This differed from the above in that the retinas were hardened in 4% alum solution for 1 hr. or more. The mixture was then centrifuged and the retinas washed first with 0·9% saline, and then with neutral buffer solution. Finally, the retinas were extracted with digitonin as before.

Lythgoe's method was slightly modified. Instead of an acid buffer, alum solution was used and the extraction with light petroleum omitted.

The '*sugar*' *method.* This was based on the work of Saito (1938). The retinas were shaken up with 45% sucrose solution and centrifuged. The heavy residue consisted of a grey layer of retinal debris and a top layer of black pigment epithelium. The decanted supernatant liquid contained the 'rods' in suspension and they could be thrown down by diluting the sugar solution with three to five volumes of 0·9% saline. The precipitated 'rods' were then washed free from sugar, and centrifuged, hardened in alum

and washed with neutral buffer and finally extracted with digitonin as before. Usually, but not always, the material was left in contact with alum or digitonin for 1 hr. at each stage, the digitonin solution of rhodopsin was generally mixed with an equal volume of a buffer solution and recentrifuged before the absorption spectrum was measured.

Absorption measurements

All measurements were made with a Beckman photoelectric spectrophotometer. A 1 cm. cell was used. The compensating cell contained a solution of digitonin and buffer of the same strength as that used for the rhodopsin solution.

Although rhodopsin solutions are very sensitive to light, little photodecomposition takes place while the absorption measurements are being made. The cells were filled in the dark room (red light) and placed in the cell holder, which was carried under a black cloth to the instrument and quickly placed in position in the light-tight cell space.

RESULTS

Influence of the method of extraction on the optical properties of rhodopsin solutions

Twenty-three solutions of frog rhodopsin were obtained by one or other of the four methods of extraction described above. The mean value of $P_{\lambda\ \text{min.}}$ is shown below for each of the four methods (Table 2).

Table 2. *Values of $P_{\lambda\ \text{min.}}$ for rhodopsin solutions*

Animal	Extraction procedure	$P_{\lambda\ \text{min.}}$	No. of samples
(*a*) Frogs	'No alum' method	0·87	3
	Lythgoe method	0·603	3
	'Alum' method	0·517	6
	'Sugar' method	0·360	11
	'Sugar' method		
(*b*) Frogs	'Summer' Frogs	0·394	7
	'Spring' Frogs	0·30	4
(*c*) Rats	'Alum' method	0·64	8
	'Sugar' method	0·48	8

For a probability of 0·05 the following differences in $P_{\lambda\ \text{min.}}$ would be significant; (*a*) 0·109, (*b*) 0·089, (*c*) 0·096.

Each method of extraction gives, on the average, a different degree of optical purity except that the 'alum' and the Lythgoe methods are much the same. The results using the 'sugar' method can be subdivided by considering the 'spring' frogs separately from the 'summer' frogs. (Inverted commas are used to indicate that the difference may not be due entirely to a seasonal change in frog retinas.)

Sixteen rat-rhodopsin solutions were obtained using the 'alum' and 'sugar' methods. The 'no alum' method gave solutions so impure that the ratio $E_{\lambda\ \text{min.}}/E_{\lambda\ \text{max.}}$ lacked meaning. In addition the presence of appreciable amounts of haemoglobin and impurities giving rise to turbidity emphasized the disadvantage of not using alum.

Haemoglobin was occasionally present in the 'alum' solutions, but never in the 'sugar' solutions.

For extracts made from retinas of sheep and cattle, the major difference was between the sheep rhodopsin using the 'alum' method and the bovine rhodopsin using the 'sugar' method. Bovine eyes had been tried previously, but the presence of large amounts of haemoglobin in the retina gave rise to inferior preparations. However, with the 'sugar' method, solutions of rhodopsin were obtained free from haemoglobin. With sheep retinas, solutions free from haemoglobin were only occasionally obtained, using the 'alum' method. The mean value of the ratio $P_{\lambda\ \text{min.}}$ was 0·51 for bovine rhodopsin ('sugar' method, two samples) and 0·664 for sheep rhodopsin ('alum' method, 12 samples).

The extraction of rhodopsin (or porphyropsin) from fish retinas proved to be difficult. With pike, haddock, and perch, the absorption curves exhibited much irrelevant absorption, but better results were obtained later with char.

Yield of rhodopsin and method of estimation

The amount of absorption due to rhodopsin at $\lambda_{\text{max.}}$ can be calculated in two ways.

Method 1. A correction procedure for rhodopsin can be obtained analogously to that developed by Morton & Stubbs (1946) for vitamin A. They showed that if the irrelevant absorption is a linear function of the wavelength the following formula for the corrected extinction can be deduced by geometrical reasoning:

$$E_{\text{corr.}} = \frac{(\lambda_3-\lambda_1)E_2-(\lambda_3-\lambda_2)E_1-(\lambda_2-\lambda_1)E_3}{(\lambda_3-\lambda_1)\alpha_2-(\lambda_3-\lambda_2)\alpha_1-(\lambda_2-\lambda_1)\alpha_3},$$

where E_2 is the extinction at wavelength λ_2 (near to $\lambda_{\text{max.}}$) and E_1 and E_3 are the extinctions at wavelengths λ_1 and λ_3 respectively (on either side of $\lambda_{\text{max.}}$). α_1, α_2 and α_3 are constants depending on the shape of the absorption spectrum of the pure substance.

For rhodopsin $\lambda_1 = 480$, $\lambda_2 = 500$ and $\lambda_3 = 520$ mμ. were chosen as suitable values. Substituting in the above formula, one obtains:

$$E_{\text{corr.}} = \frac{2E_2-E_1-E_3}{2\alpha_2-\alpha_1-\alpha_3} = K(2E_2-E_1-E_3),$$

where $K = \dfrac{1}{2\alpha_2-\alpha_1-\alpha_3}$ and is a constant. K can be estimated by using method 2 where applicable.

Method 2. If the rhodopsin solution is alkaline (about pH 9) then the difference in the extinction at 500 mμ., before and after bleaching, is equal to the extinction due to rhodopsin.

If it is assumed that the absorption spectrum of rhodopsin is unaffected by the pH, the value of K obtained in alkaline solutions will apply to all solutions.

K (for frog rhodopsin) $= 4{\cdot}86 \pm 0{\cdot}09$,
K (for rat rhodopsin) $= 4{\cdot}59 \pm 0{\cdot}06$.

Table 3. *Relationship between the yield of rhodopsin/sheep retina and the volume of digitonin solution used*

Average number (n) of retinas extracted/ml. of digitonin solution (v = volume used, $n/v = c$)	Yield of rhodopsin $E_{1\,cm.}/c$ (E at 500 mμ., 1 cm. cell)	No. of separate experiments
6·7	0·011	2
5·3	0·012	3
4·7	0·016	3
4·0	0·021	4
3·2	0·047	3
2·4	0·134	6
1·6	0·101	19
1·3	0·130	10
0·6	0·164	1

Table 4. *Mean yields of rhodopsin using retinas from the different species under comparable conditions of extraction*

Species	Mean yield $E_{1\,cm.}/c$ (E at 500 mμ., 1 cm. cell)	Standard deviation	No. of separate experiments
Frog	0·034	0·015	19
Toad	0·015	—	1
Guinea pig	0·003	—	1
Sheep	0·116	0·036	36
Rat	0·021	0·008	16
Goat	0·26	—	1
Ox	0·175	—	1

An absorption spectrum of pure frog rhodopsin

The 'purest' solution of rhodopsin (i.e. the one showing the least irrelevant absorption) so far obtained had $P_{\lambda\,min.} = 0{\cdot}24$. The absorption at

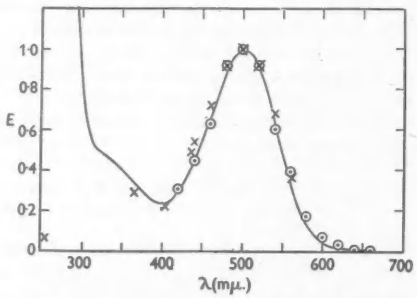


Fig. 1. —— Absorption spectrum of the 'purest' frog rhodopsin so far obtained. ⊙, points from Granit's (1947) cat scotopic dominator curve. ×, relative photochemical efficiencies $[(\epsilon_\lambda \times \gamma_\chi)/(\epsilon_{500m\mu.} \times \gamma_{500m\mu.})$ after Schneider, Goodeve & Lythgoe (1939)].

700 mμ. was 0·003 and this quantity has been subtracted from the values at all the other wavelengths. This, of course, is an underestimation of the absorption due to the scattering of light (p. 8), but the true value will not be less than 0·003. The resulting absorption spectrum is shown in Fig. 1 with the extinctions at all wavelengths expressed as a fraction of that at $\lambda_{max.}$ (500 mμ.).

Another solution which was unusually pure was char (*Salvelinus willoughbii*) 'rhodopsin', $\lambda_{max.}$ 514 mμ. and $P_{\lambda\,min.} = 0{\cdot}23$. (This species yields a mixture of porphyropsin and rhodopsin.)

Wavelength of maximum absorption in rhodopsin solutions

Scattering of light is appreciable in rhodopsin solutions; they are in fact often a little turbid. By assuming the applicability of Rayleigh's law (light scattering $E_\lambda = c/\lambda^4$) and giving to c a series of hypothetical values, a range of scattering curves can be obtained. Each of these can be superimposed on the experimental curve in which $P_{\lambda\,min.}$ is least.

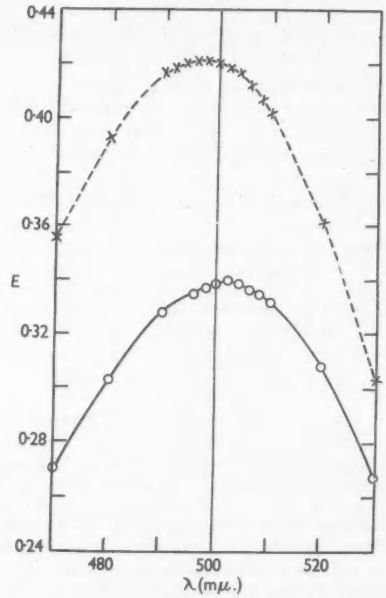


Fig. 2. Comparison of $\lambda_{max.}$ for frog and rat rhodopsin. ×, rat. ○, frog.

The results will be curves corresponding with 'impure' rhodopsin solutions in which the 'impurities' are responsible for light scattering conforming to the Rayleigh Law. Inspection of such curves, shows that if in the summation curves $P_{\lambda\,min.}$ is $< 0{\cdot}32$, the position of $\lambda_{max.}$ is not appreciably different from that observed for the best preparations. When $P_{\lambda\,min.}$ is $> 0{\cdot}33$ but $< 0{\cdot}45$ the displacement is about 1 mμ.; if $P_{\lambda\,min.}$ is $> 0{\cdot}45$ but $< 0{\cdot}55$, it is 2 mμ. and when the ratio is $> 0{\cdot}56$ but $< 0{\cdot}63$ it is 3 mμ.

Table 5. *Positions of $\lambda_{max.}$ for rhodopsin preparations after correcting for the effect of scattering*

	$P_{\lambda\ min.}$	$\lambda_{max.}$ observed (mμ.)	$\lambda_{max.}$ corr. (mμ.)
Frog	0·40	502	503
	0·38	502	503
	0·32	502	502
	0·24	503	503
	0·35	501	502
	0·32	502	502
	0·36	502	503
		Mean **502**	**502·6**±0·5
Rat	0·54	496	498
	0·49	496	498
	0·46	496	498
	0·49	697	499
	0·39	497	498
	0·41	497	498
	0·63	495	498
	0·54	696	498
	0·46	497	499
		Mean **496·5**	**498·2**±0·4
Ox	0·49	498	500
	0·50	487	499
Sheep	0·38	498	499
	0·42	498	499
Haddock	0·93	approx. 500	—
Char	0·23	514	514
	0·49	512	514
Pike	0·86	approx. 525	—
Perch	0·82	524	—
	0·74	528	—
	0·83	520	—

— Means that correction for irrelevant absorption is of doubtful value.

Ultraviolet absorption shown by rhodopsin solutions

As rhodopsin is a conjugated protein, the observed ultraviolet absorption will be a summation of three contributions: (i) that due to the prosthetic grouping, (ii) that due to selectively absorbing amino-acid residues in the carrier protein, and (iii) that due to impurities including proteins other than rhodopsin.

All preparations show selective absorption in the region 260–280 mμ., but those which exhibit low values for $P_{\lambda\ min.}$ tend to have the lowest intensity of absorption in the ultraviolet region.

If the absorption spectrum of a pure protein, excluding nucleoproteins, is considered, the curve is found to agree very well with a summation of contributions due to the amounts of tyrosine, tryptophan and phenylalanine, found to be present by ordinary methods of analysis of protein hydrolysates.

The ultraviolet absorption maximum shown by the best rhodopsin preparations occurs near 270 mμ. (pH 9) and a preliminary consideration of its origin is worth while.

An approximate estimate of the absorption due to tyrosine and tryptophan was made in the following rather circuitous manner. The absorption due to rhodopsin at 500 mμ. was determined by the three-point fixation formula. By using the absorption spectrum of the purest rhodopsin solution so far obtained, the absorption at 360 mμ. due to rhodopsin could be calculated. This wavelength was chosen as probably the shortest wavelength in the standard curve at which the irrelevant absorption was negligible. By subtracting the absorption shown by the best rhodopsin at 360 mμ. from the total absorption, the amount of the irrelevant absorption at that wavelength could be obtained. By assuming that the irrelevant absorption was due to scattered light and obeyed Rayleigh's Law, the irrelevant absorption at 290 and 280 mμ. could then be calculated. If it be assumed that the only absorption at 280 mμ. and 290 mμ. is that due to tyrosine, tryptophan and irrelevant contaminants, then the amounts of tyrosine and tryptophan can be calculated according to the procedure of Goodwin & Morton (1946). Their data have been modified so as to apply to the wavelengths 290 and 280 mμ. and at a pH of 9·3.

E_{280} is the extinction at 280 mμ. after subtracting the irrelevant absorption, and similarly for E_{290}. If x is the extinction at 280 mμ. due to tyrosine and y is

Table 6. *A trial analysis of the ultraviolet absorption band of rhodopsin solutions*

$P_{\lambda\ min.}$ (approx. 400 mμ.)	$\lambda_{max.}$ ultraviolet region (mμ.)	*$P_{\lambda\ max.}$ (approx. 270 mμ.)	$P_{280\ m\mu.}$ (corrected for irrelevant absorption)	$P_{280\ m\mu.}$ (due to tyrosine)	$P_{280\ \mu m.}$ (due to tryptophan)
0·32	277	3·7	3·4	1·6	1·8
0·37	272	5·7	4·5	2·3	2·2
0·46	269	5·8	4·8	2·2	2·6
0·52	267	21·4	13·3	8·5	4·8
0·56	267, ~280	14·3	10·2	7·2	2·9
0·66	265	8·6	6·9	5·1	1·8
0·70	270	9·2	7·2	4·3	2·9
0·73	266	24·6	19·4	10·9	8·5
0·77	270	6·3	4·4	1·5	2·9
0·79	275	22·8	20·7	11·9	8·8
0·92	~272	11·7	11·3	3·9	7·4

* i.e. $E_{270\ m\mu.}/E_{500\ m\mu.}$

~ denotes inflexion.

the extinction at 280 mμ. due to tryptophan then the following formulae hold at pH 9·3.

$$E_{280} = x + y,$$
$$E_{290} = 0{\cdot}442x + 0{\cdot}791y.$$

Further, knowing x and y and the full absorption curves of tyrosine and tryptophan at pH 9·3 it is possible to calculate the absorption due to those two substances at any other wavelength. If the absorption so calculated as due to the tyrosine and tryptophan content of the solution, is subtracted from the total absorption a difference curve is obtained. This curve always had an inflexion or maximum at about 260 mμ. (Fig. 3).

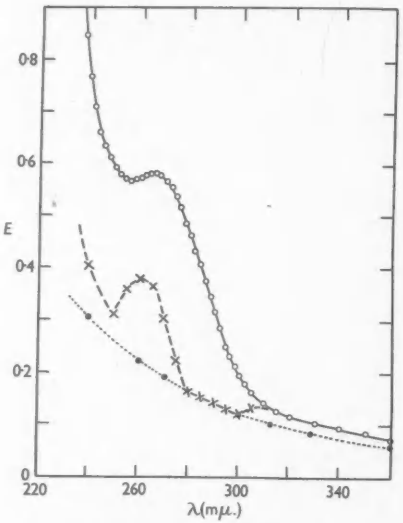


Fig. 3. Attempted analysis of rhodopsin absorption in the region 240–300 mμ. O–O–O–O, observed curve; ....., calculated irrelevant absorption assumed due to scattering; ×–×–×–×, difference curve after subtracting absorption attributable to tyrosine and tryptophan (note 260 mμ. maximum).

Absorption due to 'impurities' generally

This absorption, down to about 260 mμ., could be calculated as follows: the corrected absorption at 500 mμ. due to rhodopsin could be calculated by the three-point correction procedure and then by using the data for the purest rhodopsin the absorption due to rhodopsin at any particular wavelength could be calculated. When this was subtracted from the original absorption the spectrum of the impurities could be obtained (Fig. 4).

DISCUSSION

In 1942 Hecht had stated that the rhodopsin solutions obtained by Wald (1938), Lythgoe (1937), Krause & Sidwell (1938), Saito (1938) and Chase & Haig (1938), were substantially pure and that the absorption spectrum had been adequately characterized. One of the purposes of the present paper is to show that pure rhodopsin has not yet been

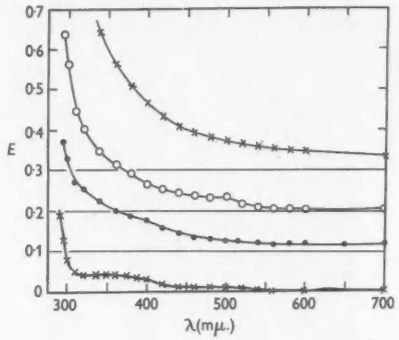


Fig. 4. Example of irrelevant absorption in different frog rhodopsin preparations obtained by subtracting the 'best' curve from the observed curves. (Scale displaced to avoid overlapping.)

obtained. The ratio $P_{\lambda\ \text{min.}}$ for the purest solution previously obtained was 0·30 (Lythgoe, 1937), but in the present work a value of 0·24 was obtained.

It is evident from the results obtained with different methods of extraction, that the sugar method for separating the outer segments of the rods, followed by hardening with alum, is the best method. Even then, however, the value obtained for $P_{\lambda\ \text{min.}}$ is very variable. The poor results obtained with Lythgoe's (1937) method may be due to insufficient experience of it, but the fact that superior results were obtained with the sugar method under comparable conditions confirms the efficacy of the latter. Bliss (1948) has independently used a similar method of extraction for cephalopsin.

The unexplained difference between the 'spring' and 'summer' frogs and the great variability in the purity of successive preparations, obtained by the same method, demonstrates that there is some factor which has so far eluded control.

The porphyropsin and rhodopsin solutions obtained from fish were, with the exception of char, very impure. This seemed to be due to the large amounts of melanin associated with the pigment epithelia which were very often difficult if not impossible to separate from the retina.

The influence of the ratio of the volume of digitonin solution to the volume of retinal tissue being ex-

tracted is interesting. It can be shown that for a given volume of retinal tissue there is a minimum volume of digitonin below which the extraction is not quantitative. One of the chief advantages of the sugar method is that the volume of tissue is reduced to a very small amount and better extraction occurs.

An examination of the results presented above enables some conclusions to be drawn concerning the nature of the impurities. The absorption curves shown in Fig. 4 represent an approximation to that of the 'impurities'. The effect of colloidal particles in scattering the light is shown by the gradual rise in absorption from 700 mμ. to the ultraviolet. This portion of the absorption curve does not obey Rayleigh's Law exactly, but the divergence is not great. From this it can be concluded that the particles are not greatly different in size from the wavelength of visible light (0·4–0·7 μ.) (cf. Oster, 1948).

However, below about 400 mμ. the smooth curve due to the impurities is broken by an abrupt increase. This must be due to specific absorption and will correspond to the 'yellow impurities' of Chase & Haig (1938) and others.

Although the figures in Table 6 are only approximate, three facts emerge clearly: (1) the ratio of the intensity of the protein absorption to that due to rhodopsin is very variable and strongly suggests that some of the 'impurity' is protein in nature. (2) An approximate estimate of tyrosine and tryptophan content, calculated as a percentage of the rhodopsin molecule, can be obtained as follows.

$E^{1\%}_{1\,\mathrm{cm.}}$ (500 mμ.) for rhodopsin is about 6·6 (calculated from the data of Broda, Goodeve & Lythgoe (1940).

$E^{1\%}_{1\,\mathrm{cm.}}$ (280 mμ.) for tyrosine (pH 9·3) is 78 and for tryptophan 262.

$$\text{Hence } \%\text{ tyrosine} = \frac{P_{280}\text{ (due to tyrosine)}}{78} \times \frac{6{\cdot}6}{1{\cdot}0} \times \frac{100}{1}$$

and

$$\%\text{ tryptophan} = \frac{P_{280}\text{ (due to tryptophan)}}{262} \times \frac{6{\cdot}6}{1{\cdot}0} \times \frac{100}{1}.$$

The rhodopsin in the solution with $P_{\lambda\,\mathrm{min}} = 0{\cdot}32$ appeared to have 14 % tyrosine and 5 % tryptophan. In contrast the rhodopsin in the solution with $P_{\lambda\,\mathrm{min.}} = 0{\cdot}79$ appeared to have 102 % tyrosine and 22 % tryptophan, which simply means that the solution is grossly impure. As most proteins have less than 5 % tryptophan and not much more tyrosine this is very strong evidence for the presence of protein impurities or large amounts of tyrosine-containing material. (3) The absorption at 270 mμ. cannot satisfactorily be accounted for in terms of tyrosine and tryptophan. The band at 260 mμ. which is deduced could be due to purines and/or pyrimidines, perhaps as nucleic acids.

From these results and from previous work the following conclusion can be drawn concerning the nature of the impurities. Colloidal particles cause general absorption, approximately obeying Rayleigh's Law and 'yellow impurities' cause absorption below 400 mμ. This absorption may be due to phospholipin breakdown products (Broda, 1941, and Ishimoto & Wald, 1946), protein (including nucleoprotein) and melanin. This last is rendered likely by the apparently large amounts of tyrosine present and by the fact that melanin is found in neighbouring tissues such as the pigment epithelium. There is good evidence that all three of these impurities are present but it is doubtful whether phospholipins, unless oxidized, would contribute any specific absorption.

A criterion for pure rhodopsin must be that the shape of the absorption spectrum is unaltered in any wavelength region by further 'purification'. As some of the impurities may be transparent it will be necessary to employ other criteria in addition to the spectroscopic one.

Although pure rhodopsin has not yet been obtained some deductions may be made concerning it. The ratio $P_{\lambda\,\mathrm{min.}}$ is not greater than 0·24 and a lower limit can be set in two ways. If the prosthetic group of rhodopsin contains vitamin A or retinene, then a resemblance in absorption spectrum should be apparent. Both pure vitamin A and pure retinene have a minimum and the ratio $P_{\lambda\,\mathrm{min.}}$ is 0·20 for vitamin A and 0·24 for retinene dissolved in digitonin solution. Goodeve, Lythgoe & Schneider (1942) showed that the ratio of the photosensitivity at 400 mμ. to that at 500 mμ. for rhodopsin was 0·22. The photosensitivity is the product of the molecular extinction coefficient and of the quantum efficiency and hence this ratio should correspond to $P_{\lambda\,\mathrm{min.}}$. Therefore 0·20 can be taken as the lower limit. This suggests that our purest rhodopsin solution is not far from being 'optically' pure from 400–500 mμ. In Fig. 1 it is compared to Granit's (1947) scotopic luminosity curve and with the photosensitivity curve of Goodeve *et al.* (1942).

It is proposed in a further communication to consider the absorption in the region 310–390 mμ. which decreases considerably as the protein absorption at 270 mμ. is reduced. The purer the solution the more apparent is the inflexion at 340 mμ. in the rhodopsin spectrum.

The fact that $\lambda_{\mathrm{max.}}$ varies from species to species is not entirely unexpected (cf. haemoglobin). The difference is small but quite definite; 503 mμ. for frogs to 498 mμ. for rats.

Conclusions

The approach to pure rhodopsin will have to be made in stages. The first stage is clearly to obtain a reproducible absorption curve over the region 400–700 mμ. This has been very satisfactorily attained since there is good agreement between the electrophysiological, photochemical and spectro-

photometric data, and interspecies differences are not large.

Such rhodopsin preparations could however be quite variably contaminated with colourless substances such as proteins and possibly small amounts of indicator yellow and/or vitamin A, as well as purine or pyrimidine bases. The second stage must be to establish constancy in the spectrum between 300 and 400 mμ., and a third stage to establish a constant value between the absorption at 250–280 mμ. due to the carrier protein, and the absorption at 500 mμ.

Neither stage 2 nor 3 has yet been reached and nothing is known for certain about interspecies differences.

SUMMARY

1. Eyes from frogs, rats, sheep, cattle and several species of fish have been examined as sources of visual purple.

2. A combination of Saito's (1938) sugar flotation method for separating the outer segments of the rods from the retina with alum hardening of the rods proved to be superior to the other methods tried.

3. The major impurities seem to be protein (other than the rhodopsin moiety), melanin and phospholipin.

4. The ultraviolet absorption cannot be entirely accounted for as being due to tyrosine and tryptophan. A band at 260 mμ. seemed to be present and could be due to purine or pyrimidine or both, perhaps as nucleic acid.

5. The absorption maximum ($\lambda_{max.}$) for frog rhodopsin was 503 mμ., for rat 498 mμ. and sheep and ox at 500 ± 1 mμ. Char 'rhodopsin' had $\lambda_{max.}$ at 514mμ. Perch and pike porphyropsin had $\lambda_{max.}$ at 520–530 mμ., haddock rhodopsin at approx. 500 mμ.

6. A solution of frog rhodopsin has been obtained with the extinction ratio $E_{min.}/E_{max.} = 0{\cdot}24$ and hence is considerably purer than anything so far reported.

One of us (F. D. C.) participated in this work as a holder of a New Zealand National Research Scholarship. We are indebted to the Medical Research Council and the Ministry of Food for grants.

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Studies on Rhodopsin

2. INDICATOR YELLOW

By F. D. COLLINS and R. A. MORTON
Department of Biochemistry, University of Liverpool

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The first product formed by the action of light on rhodopsin is apparently 'transient orange' (Lythgoe & Quilliam, 1938), a substance which is not stable at room temperature, but is transformed to 'indicator yellow'. As its name implies, the latter is pH sensitive; it is pale yellow in alkaline solutions and much more deeply yellow ($\lambda_{max.}$ 440 mμ.) in acid solutions (Lythgoe, 1937).

By extracting freshly bleached retinas with light petroleum, Wald (1934, 1935, 1936) obtained a new material which he called retinene. Later Morton & Goodwin (1944) showed retinene to be vitamin A aldehyde. The relationship between retinene and indicator yellow remained obscure until work in this laboratory (Ball, Collins, Morton & Stubbs, 1948 and Ball, Collins, Dalvi & Morton, 1949) showed that

retinene would combine with many amines, etc. to form compounds closely analogous to indicator yellow.

The purpose of the present paper is to examine more closely the relationship between retinene and indicator yellow on the one hand and between indicator yellow and rhodopsin on the other.

METHODS

Rhodopsin solutions were obtained by the methods discussed in the preceding paper (Collins & Morton, 1950). The pigment was destroyed photochemically ('bleached') by placing the cell containing the solution some 10–12 cm. in front of a 60 W. lamp. The absorption at 500 mμ. was measured at intervals and the bleaching continued until the extinction at 500 mμ. fell to a constant low value.

Retinal protein solutions. (*a*) Whole retinas are bleached (whereby rhodopsin is replaced by retinene which is slowly reduced to vitamin A), the tissue is hardened in alum solution (4%, w/v) and extracted with 2% (w/v) aqueous digitonin, and centrifuged. The supernatant liquid is colourless (except occasionally for traces of haemoglobin) and free from retinene but contains protein. (*b*) Rods obtained by the sugar-flotation method are bleached by light, exhaustively extracted with light petroleum to remove retinene, hardened with alum and extracted with 2% (w/v) aqueous digitonin and centrifuged. The clear liquor contains retinal protein.

Retinene$_1$ in aqueous digitonin solutions. Retinene$_1$ previously prepared in this laboratory by oxidation of vitamin A was used either as the crystalline material or a very rich concentrate. It was dissolved in aqueous digitonin (2%, w/v), the final concentration of retinene being about 10^{-5} molar, either (1) by adding the required amount of digitonin solution to solid retinene and bringing the solution to the boil or (2) by dissolving the solid retinene in a few drops of absolute ethanol before adding the digitonin solution.

As the solutions are appreciably photosensitive the process of dissolution is best carried out in a dark room with subdued or red lighting.

Alum solution. Potash alum [$K_2SO_4.Al_2(SO_4)_3.24H_2O$] was dissolved in water (4%, w/v).

RESULTS

Irrelevant absorption. All rhodopsin solutions exhibit some absorption additional to that due to the pigment itself. The 'irrelevant' contribution is itself composite and variable. The wavelength 700 mμ. is outside the range covered by appreciable rhodopsin absorption and the *E* value at that point can be used in estimating the amount of scattered light. It often happens that as a result of bleaching rhodopsin solutions, there is an appreciable improvement in transparency in the red part of the spectrum. This complication adds to the difficulty of making strictly quantitative comparisons between unbleached and bleached solutions.

$\lambda_{max.}$ of bleached rhodopsin solutions. The observed absorption curve is actually a summation of con-

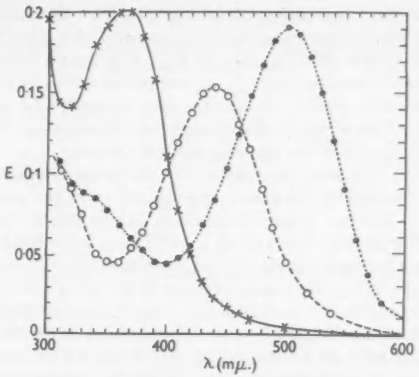


Fig. 1. Indicator yellow preparation from frog rhodopsin. ·●··●·, Rhodopsin; –○—○–, acid indicator yellow; —×—×—, alkaline indicator yellow. (All curves have had irrelevant absorption due to scattering subtracted.)

Table 1. *Observed and 'corrected' values for the wavelengths of maximum absorption shown by indicator yellow*

pH	$\lambda_{max.}$ obs. (mμ.)	$\lambda_{max.}$ corr. (mμ.)
(*a*) Bleached frog-rhodopsin preparations		
Excess alkali	360	365
9·2	~approx. 360	365
9·14	~approx. 360	365
8·05	363	367
8·0	367	367
8·0	360	365
7·6	367	373
7·5	367	367
7·4	367	367
7·4	360	370
7·0	365	367
1·76	435	440
Excess acid	435	440
(*b*) Bleached rat-rhodopsin preparations		
9·3	360	367
9·2	357	367
9·0	363	367
7·7	365	370
7·4	370	377
7·4	377	383
7·2	360	370
7·0	~370	375
7·0	365	373
6·66	~366	370
6·5	370	385
5·2	~370	367
4·0	400	417
Excess acid	435	440
	435	440
	405	435
	400	435
	430	440
	410	440

~ denotes inflexion.

tinuous absorption increasing with decreasing wavelength, and the roughly symmetrically selective absorption on either side of $\lambda_{max.}$ of indicator yellow itself. The observed $\lambda_{max.}$ as compared with the true indicator yellow maximum may therefore be displaced as a result of the irrelevant absorption. The displacement can be estimated as follows.

(*a*) The absorption spectrum of a given solution of unbleached rhodopsin is determined over the range 250–700 mμ. Using the fixation points 480, 500 and 520 mμ. the observed $E_{max.}$ (500 mμ.) is reduced to a corrected value (Collins & Morton, 1950).

(*b*) From the absorption curve for the 'reference' preparation of rhodopsin, i.e. the solution exhibiting the highest 'optical' purity so far obtained (with E_λ expressed as a fraction of $E_{max.}$) the whole curve from say 340–600 mμ. for the solution under study can be calculated by simple proportion given $E_{corr.}$ at 500 mμ.

(*c*) From the original observed curve (*a*) the corrected curve is subtracted ($E_{obs.}-E_{corr.}$) at wavelengths 5 or 10 mμ. apart. The difference curve represents irrelevant absorption.

(*d*) If it could be assumed that after bleaching the irrelevant absorption was substantially the same, the 'true' indicator yellow absorption would be obtained by subtracting the irrelevant absorption curve from the observed 'bleached' curve.

This procedure leads to 'corrected' values for $\lambda_{max.}$ and the best evidence for its validity is the constancy of the results.

Retinene and proteins. The general approach is illustrated by the following qualitative experiment.

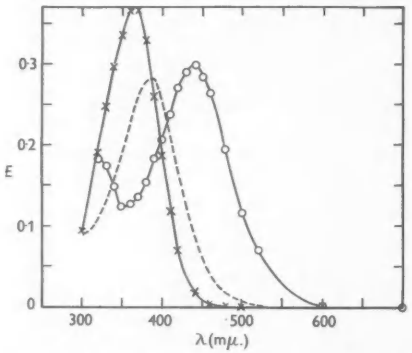


Fig. 2. Retinene and retinal protein. ----, Retinene and neutral protein solution; — × — × —, same solution plus alkali; –○—○–, previous solution plus excess acid.

To a solution of egg albumin in water is added a few drops of a solution of retinene in ethanol and the mixture is divided into two portions (*a*) and (*b*); (*a*) is acidified, but no change in colour occurs, (*b*) is made alkaline and the colour becomes paler, it is then acidified and becomes orange in colour; the acidified solution (*a*) is made alkaline and then acid and the orange colour appears.

For more quantitative experiments a neutral solution containing protein from sheep retinas may be used. When retinene is added $\lambda_{max.}$ occurs at 387 mμ. On making the solution alkaline $\lambda_{max.}$ occurs at 365 mμ., and subsequent acidification brings about the appearance of an absorption band with $\lambda_{max.}$ 440 mμ. Under such circumstances the relative intensities of absorption are: retinene ($\lambda_{max.}$ 387 mμ.) $E_{max.}$ 1·0; acid indicator yellow ($\lambda_{max.}$ 440 mμ. $E_{max.}$ 1·0–1·05; alkaline indicator yellow ($\lambda_{max.}$ 370 mμ.) $E_{max.}$ 1·26–1·43.

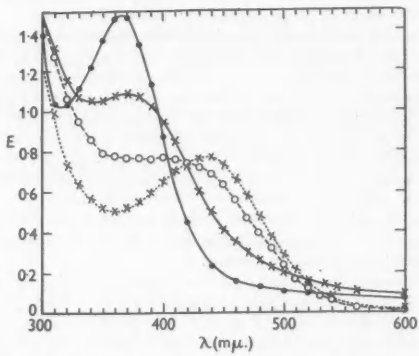


Fig. 3. Indicator yellow absorption curves uncorrected for irrelevant absorption, but with the absorption at 500 mμ. due to rhodopsin put equal to 1·00 (rhodopsin curve not shown). –●—●–, pH 8·0; – × — × –, pH 6·5; –○–○–, pH 4·0; ··· × ···· × ···, excess HCl. The isosbestic point is only apparent, because of the differences in irrelevant absorption.

The *E* values are somewhat variable as the amount of irrelevant absorption varies from one experiment to another.

If acid is added in excess to the solution showing $\lambda_{max.}$ 370 mμ. obtained by adding alkali to the retinene-protein solution, an orange precipitate is formed which can be centrifuged down and washed with water. On vigorous shaking with ethanol some of it dissolves and the solution exhibits $\lambda_{max.}$ 445 mμ.

Further characteristics of the absorption spectrum of indicator yellow. One solution (Table 1) of rat rhodopsin (pH 4) had $\lambda_{max.}$ at 417 mμ. immediately after bleaching, but on standing for 4 hr. in the dark, the maximum disappeared and only an inflexion at about 360 mμ. remained.

Other solutions of acid indicator yellow ($\lambda_{max.}$ 440 mμ.) showed on standing a drift of $\lambda_{max.}$ from 440 to 430 mμ., but if the solution was first made alkaline and then acid again $\lambda_{max.}$ reappeared at 440 mμ. This will be referred to in the discussion.

Table 2. *Quantitative relationship in the conversion of rhodopsin to indicator yellow*

(The change ($\Delta E_{500\ m\mu.}$) in intensity of absorption at 500 mμ. measures the destruction of rhodopsin on complete bleaching and the rise ($\Delta E_{370\ m\mu.}$) in absorption at 370 mμ. measures the formation of indicator yellow.)

Species	pH of solution	ΔE at 700 mμ.	ΔE at 370 mμ.	ΔE at 500 mμ.	$\Delta E_{370 m\mu.}/\Delta E_{500 m\mu.}$
Frog	9·2	−0·005	0·116	−0·152	0·76
	9·14	−0·000	0·142	−0·189	0·75
Rat	9·2	−0·002	0·086	−0·117	0·74
	9·3	−0·003	0·135	−0·180	0·75
	9·0	−0·000	0·274	−0·355	0·77

For frog-rhodopsin solution at pH 1·76, $\Delta E_{500\ m\mu.}$ was 0·189, and $\Delta E_{440\ m\mu.}$ 0·070 and $\Delta E_{700\ m\mu.}$ was not measurable, so that $\Delta E_{440\ m\mu.}/\Delta E_{500\ m\mu.}=0·37$.

Extraction of retinene from retinas. A moist rod preparation (Collins & Morton, 1950, previous paper) from 12 dark-adapted frogs was first thoroughly extracted with light petroleum and then the rhodopsin was bleached. Retinene could then be extracted with light petroleum, but only very slowly.

The moist rods were dealt with in a dark room with a red light. They were placed in a centrifuge tube and shaken vigorously with light petroleum (5 ml.) and centrifuged. The liquid was poured off and replaced by a fresh portion (5 ml.) of solvent and the process repeated. In all, 10 such treatments were given in the dark room spread over a working day. Each 'washing' with light petroleum was examined for ultraviolet absorption. This was at first considerable though unselective, and the final solution was practically transparent. The residual rod preparation was then bleached by a strong light and at once re-extracted twice with light petroleum. The yield of retinene determined spectrophotometrically was 0·5 μg. After 3 hr. a second (double) extraction gave 1·0 μg. and after 5 hr. a third (double) extraction gave 0·3 μg.

Several other experiments with frog rod preparations led to the same conclusion, namely that after bleaching less retinene was obtained in the first extractions than in those made 1–2 hr. after exposure to light.

On the other hand if whole, fresh, dark-adapted frog retinas were suspended in light petroleum and exposed to light the retinene at once went into solution.

Retinene was not, however, extracted from ox or rat retinas treated similarly.

Extraction of indicator yellow from dried haddock retinas. Through the courtesy of the Director of the Torry Research Station and of Dr J. A. Lovern, one of us (F. D. C.) was able to keep live haddock in tanks so as to secure at any rate partial dark adaptation.

Alum-treated retinas were dried *in vacuo* and were found to keep fairly well. A portion of the dried material was extracted with absolute ethanol and the solution exhibited the absorption curves shown in Fig. 4. These results are discussed on p. 16.

Extraction of retinene from bleached rhodopsin solutions. It cannot be assumed without investigation that rhodopsin-containing solutions are free from vitamin A, which might indeed be present in extracts from incompletely dark-adapted retinas, especially if the enzyme-destroying alum treatment had been delayed or omitted. Under such circumstances bleached rhodopsin solutions might contain both retinene and vitamin A.

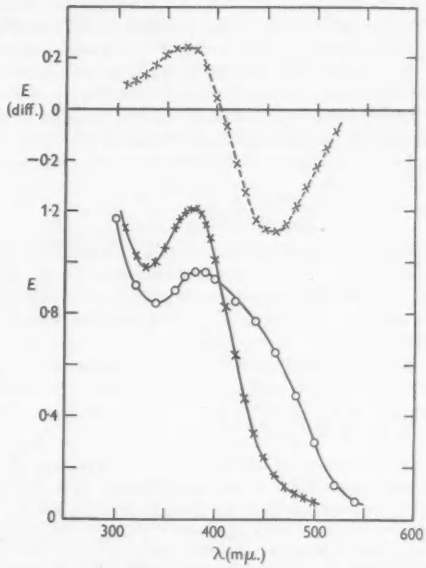


Fig. 4. −○—○−, Ethanol extract of dried haddock retinas; −×—×−, the same extract after 1 hr.; −×---×−, a difference curve illustrating the disappearance of a maximum near 450 mμ., and its replacement by a maximum near 375 mμ.

It is possible to extract retinene from bleached rhodopsin solutions readily by means of a mixture of ethanol and light petroleum, but Bliss (1948) has shown that light petroleum alone is effective if emulsions are broken down by strong centrifuging.

This we confirm, but the extracts obtained with or without added ethanol are very impure as judged by ultraviolet absorption or by the antimony trichloride colour test. If, however, the light petroleum extract is taken to dryness and left in contact with 5% ethanolic potassium hydroxide for 0·5 hr. at room temperature and the retinene is re-extracted (chloroform) the 664 mμ. maximum in the colour test appears very clearly without turbidity (cf. Bliss, 1948). This, again, is confirmed. Three typical rhodopsin solutions gave 45, 75 and 44% recoveries of retinene at the pH values 7·4, 9·3 and 6·5 respectively, assuming that in aqueous digitonin the molecular extinction coefficients of rhodopsin at 500 mμ. and retinene at 380 mμ. were respectively 50,000 (see Discussion) and 40,000. Vitamin A was not, however, present in quantity.

In cases where vitamin A and retinene are both present the following procedure is useful. The band at 617 mμ. due to vitamin A reaches its maximum intensity after 15 sec. and that at 664 mμ. due to retinene after 100 sec. For pure vitamin A the ratio of the extinction at 664 mμ. (100 sec.) to that at 617 mμ. (at 15 sec. after mixing) is 0·094. (This figure is due to Dr J. Glover.) For retinene the ratio of the extinction at 617 mμ. (15 sec.) to that at 664 mμ. (100 sec. from mixing) is 0·560.

Hence if E_{617} is the extinction at 617 mμ. at 15 sec. and E_{664} is the extinction at 664 mμ. at 100 sec., the following equations will hold:

$$E_{664} = E_R + 0{\cdot}094E_A,$$
$$E_{617} = 0{\cdot}560E_R + E_A$$

where E_R is the extinction at 664 mμ. (100 sec.) due to retinene and E_A is the extinction at 617 mμ. (15 sec.) due to vitamin A. $E^{1\%}_{1\,cm.}$ (617 mμ.) for vitamin A is 5000 and $E^{1\%}_{1\,cm.}$ (664 mμ.) for retinene is 4000.

Bleached rhodopsin and p*-aminobenzoic acid.* *p*-Aminobenzoic acid reacts readily with retinene to give a band at 535 mμ. in acid solutions (Ball, Goodwin & Morton, 1948).

The following four experiments were carried out. (1) *p*-Aminobenzoic acid in excess was added to freshly bleached slightly acid rhodopsin and the solution was made first alkaline and then acid. (2) *p*-Aminobenzoic acid in excess was added to a slightly acid rhodopsin solution, which was then bleached, made alkaline and finally acid. (3) *p*-Aminobenzoic acid in excess was added to a freshly bleached neutral rhodopsin solution, which was then acidified. (4) *p*-Aminobenzoic acid was added in excess to a neutral rhodopsin solution, which was then bleached and made acid.

All these experiments were negative in the sense that no band at 535 mμ. was obtained, although *p*-aminobenzoic acid reacted readily with retinene in aqueous digitonin to give the 535 mμ. maximum. These experiments will be referred to in the discussion.

Retinene reductase (*vitamin A dehydrogenase*). A few experiments were carried out which showed that crystalline retinene was converted to vitamin A in the presence of fresh sheep retinas. However, the conversion was not quantitative and most of the retinene gave rise to products which had no specific absorption.

In one experiment three preparations were used as follows. (1) Three light-adapted retinas were covered with a solution of retinene in 2% (w/v) aqueous digitonin. (2) Three light-adapted retinas were kept at 100° for 5 min. and then covered with a solution of retinene in aqueous digitonin. (3) Three light-adapted retinas were left untreated.

The three preparations were left for 2 hr. and were then dried with anhydrous Na_2SO_4 and extracted with light petroleum. This solvent was removed *in vacuo* and replaced by 10 ml. *cyclo*hexane. The amounts of vitamin A were estimated by the three-point correction procedure of Morton & Stubbs (1946).

No trace of retinene could be found (absence of $\lambda_{max.}$ or inflexion at 370 mμ. and of a maximum at 664 mμ. with the antimony trichloride reagent).

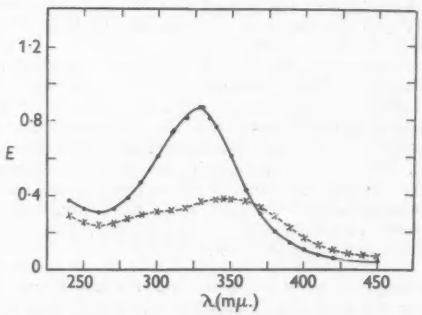


Fig. 5. –– × –– × ––, Retinene after incubation with boiled retinas; ———, after incubation with fresh retinas. Much retinene has been destroyed in the absence of retinene reductase, but with the fresh retinas the absorption curve is mainly due to vitamin A.

The amounts of vitamin A were as follows: (1) 4·0 μg.; (2) 0·5 μg. and (3) 0·8 μg. Approximately 18 μg. of retinene had been added to (1) and (2). Therefore in (1) 18% of the retinene had been converted to vitamin A while 82% was unaccounted for. Fig. 5 shows the absorption curves obtained in another experiment.

DISCUSSION

In favourable circumstances the irrelevant absorption is not changed as a result of irradiation, but in some cases careful comparison of intensities of absorption, particularly on the long-wave side of 650 mμ., indicates a change in the light loss due to scattering.

A decrease in the size or number of colloidal particles would be needed to account for the decreased scattering (cf. Oster, 1948) which in one instance was followed by measuring the change in absorption from 600 to 1000 mμ. The immediate importance of this variable phenomenon is that quantitative interpretation must rest on selected absorption curves where the change in scattering is very small (Table 2).

The position of the absorption maximum of acid indicator yellow at 440 mμ. is well verified within narrow limits, but that of alkaline indicator yellow has not so often been recorded.

The values of the two maxima observed in the present work (440 and 365 mμ.) were in good agreement, whether the indicator yellow came from rat or frog retinas, provided that the pH was definitely on the acid or alkaline side. For more nearly neutral solutions the results were more variable (cf. Table 1), especially for rat preparations.

Now in a simple two-component system with maxima at 440 and 365 mμ., the bands being of the same order of intensity and half-width, there should clearly be an isosbestic point. When samples of the same indicator yellow solution were examined at different pH values no definite isosbestic point could be recorded (cf. Lythgoe, 1937). Further, trial summations of the two curves show that in a two-component system there would be two distinct maxima over a considerable range of mixtures (cf. Fig. 6). In fact, in no case has bleached rhodopsin solution been obtained which had more than one maximum.

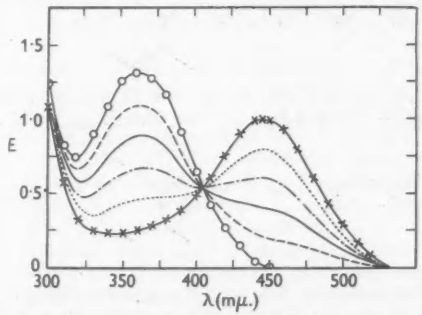


Fig. 6. Theoretical mixture curves for acid and alkaline indicator yellow in various proportion. Note the absence of an intermediate $\lambda_{max.}$ in the summations.

It must therefore be concluded that freshly bleached solutions of rhodopsin contain a third absorbing component, retinene itself being by far the most likely.

Mixtures of retinene ($\lambda_{max.}$ 385 mμ., ethanol) and alkaline indicator yellow ($\lambda_{max.}$ 365 mμ.) will have a single shifting maximum intermediate between 385 and 365 mμ. because the curves overlap more than those of the two forms of indicator yellow. Therefore the position of $\lambda_{max.}$ in many neutral solutions could be accounted for if a mixture of retinene and alkaline indicator yellow were present. Now the extinction of alkaline indicator yellow at 365 mμ. is 1·26–1·43 times that of retinene at 385 mμ. (p. 12). If a neutral bleached rhodopsin solution with $\lambda_{max.}$ between 375 and 385 mμ. is made alkaline, $\lambda_{max.}$ shifts to about 365 mμ. and the intensity of absorption increases, a fact which suggests that alkali forms indicator yellow from retinene.

The solution in which $\lambda_{max.}$ was 417 mμ (Table 1) could have contained a mixture of acid indicator yellow and retinene, but the evidence is inconclusive.

The reactions of retinene throw light on the whole problem. Retinene will only combine with proteins or aliphatic amines, e.g. methylamine (cf. Ball *et al.* 1949) in an alkaline medium and, if the retinene is about 10^{-5} molar, the amine or protein has to be present in large excess ($>10^{-3}$ molar). In alkaline solutions $\lambda_{max.}$ is at 365 mμ., as for alkaline indicator yellow. If the solution is made acid, $\lambda_{max.}$ becomes 440 mμ., as for acid indicator yellow. If the solution is neutralized, $\lambda_{max.}$ tends towards 385 mμ.; $\lambda_{max.}$ at 440 mμ., would not be obtained unless the neutralized solutions are first made alkaline and then acid.

All these facts are consistent with neutral bleached rhodopsin solutions consisting of a mixture of indicator yellow and retinene.

The fact that $\lambda_{max.}$ of acid indicator yellow gradually shifts from 440 mμ. towards 400 mμ. on standing suggests that acid indicator yellow slowly changes to retinene. Most neutral bleached rhodopsin solutions had $\lambda_{max.}$ at approx. 365 mμ., which suggests that alkaline indicator yellow is formed before and not after free retinene.

The results of extracting bleached rhodopsin with light petroleum become clearer if summarized as follows:

(1) Fresh retinas immediately after bleaching—retinene is extracted readily.

(2) Bleached rhodopsin solutions—retinene is extracted partially (30–50 %).

(3) Bleached retinas after light petroleum extraction—retinene is extracted slowly.

(4) Bleached retinas dried after alum treatment—acid indicator yellow is extracted.

(1) was with frog retinas, (2) with rat, frog and sheep rhodopsin solutions, (3) was with frog retinas and (4) was with haddock retinas. (1) was not possible with rat or bovine retinas unless ethanol was added.

The interspecies differences are not sufficiently well characterized to warrant any conclusions at present, but they seem to be genuine.

Of more immediate importance is that these facts confirm the results of Bliss (1948). In every case so

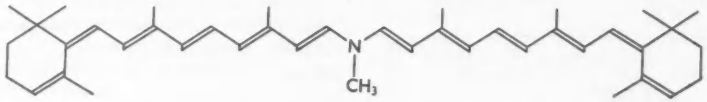
far examined the conversion of indicator yellow to retinene can be observed (or inferred), but never the reverse process. It must again be concluded that indicator yellow is formed first and may then be either wholly or partially converted to retinene.

Aminobenzoic acid reacts readily with free retinene to form a substance with $\lambda_{max.}$ at 535 mμ. The fact that *p*-aminobenzoic acid does not react with bleached rhodopsin solutions is a further indication of the absence of free retinene immediately after bleaching (p. 14).

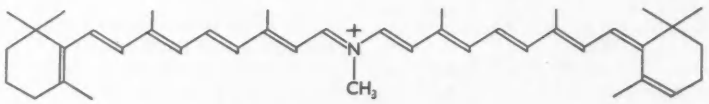
In their work on indicator yellow analogues, Ball *et al.* (1949), have shown that retinene will combine with various amines to form substances which are pH sensitive. With aromatic amines $\lambda_{max.}$ in acid is > 490 mμ. and hence natural indicator yellow cannot contain an aromatic nucleus conjugated with the amino group. Ball *et al.* (1949) conclude that, as retinene combines as readily with benzylaniline as with aniline, the resulting compounds must each contain one molecule of retinene and one of amine.

With aliphatic amines or with aromatic amines in which the aromatic ring is not joined directly to the amino group, $\lambda_{max.}$ is at 440 mμ. in acid and at 365 mμ. in alkali. Unlike the aromatic amines aliphatic amines will only react with retinene in an alkaline medium. Dimethylamine does not react but methylamine does, indicating that the most probable reaction is one between two molecules of retinene and one molecule of amine.

The simplest compound containing two molecules of retinene and one of methylamine will be:



[Note break in conjugation (cf. vitamin A_2 with $\lambda_{max.}$ 350 mμ.)] and addition of acid would give:



[Note 'full' conjugation restored (cf. carotene) with $\lambda_{max.}$ 450 mμ.]

The first structure would correspond to alkaline indicator yellow and would be expected to show $\lambda_{max.}$ 365 mμ. The second structure would correspond to acid indicator yellow and $\lambda_{max.}$ at 440 mμ. is consistent with the available spectroscopic data.

If these are truly analogues to indicator yellow its structure must be similar. Instead of having a methyl group attached to the central nitrogen atom the protein will no doubt be attached at that point.

On two occasions it has been reported that acid indicator yellow is soluble in organic solvents. In the present work (p. 13) haddock indicator yellow has been obtained in ethanol, whilst Bliss (1946) obtained a substance with $\lambda_{max} = 460$ mμ. in chloroform. Acid indicator yellow analogue prepared from β-alanine or methylamine is soluble in chloroform and $\lambda_{max.}$ is at 460 mμ. There can be very little doubt that the proposed general structure of indicator yellow is substantially correct. However, if the nitrogen is attached to a protein, it is at present difficult to see how indicator yellow can become detached.

The relationship between the values of $E_{max.}$ for rhodopsin, retinene and indicator yellow may now be discussed. Let ϵ_v, ϵ_a, ϵ_b and ϵ_r stand for molecular extinction coefficients at 500, 440, 370 and 385 mμ. respectively, for rhodopsin ($\lambda_{max.}$ 500 mμ.) acid indicator yellow ($\lambda_{max.}$ 440 mμ.) alkaline indicator yellow (λ_{max} 365–370 mμ.) and retinene ($\lambda_{max.}$ 385 mμ.) where $E = \epsilon cl$, E being extinction ($\log I_0/I$); c, molar concentration and l cell thickness, 1 cm. By 1 g.mol. is meant here $6{\cdot}06 \times 10^{-23}$ chromophoric or prosthetic groups. This definition does not prejudge the question of the number of molecules of coloured component/molecule of carrier protein.

When c and l are kept constant E_v, E_a, E_b and E_r will represent extinctions for rhodopsin, acid and alkaline indicator yellow and retinene respectively, and E_λ, e.g. $E_{500\ m\mu.}$ will represent observed values.

On bleaching an alkaline solution of rhodopsin the maximum at 500 mμ. is replaced by a maximum at 365 mμ. due to indicator yellow. The absorption at 500 mμ. initially may be written $E_{500\ m\mu.} = E_v + d$, where d represents irrelevant absorption at 500 mμ., d is assumed not to change with bleaching by light, so that (at pH 9·3) $E'_{530\ m\mu.} = d$, and $\Delta E_{500\ m\mu.} = E_v$.

Similarly the absorption at 370 mμ. before and after bleaching is written $E_{370\ m\mu.}$ and $E'_{370\ m\mu.}$. The absorption at 370 mμ. due to rhodopsin will be a constant fraction α of E_v, so that $E_{370\ m\mu.} = \alpha E_v + d'$, where d' is the irrelevant absorption. After bleaching (at pH 9·3)

$$E'_{370\ m\mu.} = d' + E_b$$

and

$$\Delta E_{370\ m\mu.} = E_b - \alpha E_v$$

and

$$\Delta E_{370\ m\mu.}/\Delta E_{500\ m\mu.} = (E_b - \alpha E_v)/E_v = E_b/E_v - \alpha.$$

From Table 2 $E_b/E_v = 0{\cdot}75 + \alpha$.

According to our best curve for rhodopsin (Collins & Morton, 1950) $\alpha = 0{\cdot}31$ which agrees well with the ratio 0·29 for the relative photosensitivities of rhodopsin at 365 and 500 mμ. (Schneider, Goodeve & Lythgoe, 1939). Accepting the latter figure

$$E_b/E_v = 1{\cdot}04.$$

Similarly it can be shown that

$$E_a/E_v = 0{\cdot}81.$$

Now let the chromophoric group (i.e. the prosthetic group responsible for the 500 mμ. maximum) of rhodopsin contain p retinene (or vitamin A, i.e. C_{20}) residues, and acid indicator yellow n such residues and alkaline indicator yellow m residues. On bleaching a rhodopsin solution of concentration c_v, the concentration of acid indicator yellow will be pc_v/n or that of alkaline indicator yellow pc_v/m. Assuming complete interconvertibility

$$E_v = \epsilon_v c_v \quad \text{and} \quad E_a = \epsilon_a c_a = \epsilon_a . pc_v/n.$$

Hence $$E_a/E_v = \epsilon_a . p\, c_v/n\epsilon_v c_v = \epsilon_a p/\epsilon_v n. \qquad (1)$$

Also $$E_r = \epsilon_r c_r'$$

(where c' and c are not equal) and

$$E_a = \epsilon_a c_a' = \epsilon_a . c_r'/n,$$

hence $$E_a/E_r = \epsilon_a c_r'/n . \epsilon_r c_r' = \epsilon_a/\epsilon_r . n. \qquad (2)$$

Similarly $$E_b/E_v = \epsilon_b . p/\epsilon_v m \qquad (3)$$

and $$E_b/E_r = \epsilon_b/\epsilon_r . m. \qquad (4)$$

It has been shown that when $E_v = 1{\cdot}00$

$$E_a = 0{\cdot}81 \quad \text{and} \quad E_b = 1{\cdot}04.$$

For the compound formed between β-alanine and retinene, a good analogue of indicator yellow (Ball *et al.* 1949), it has been found that when $E_r = 1{\cdot}00$, $E_a = 1{\cdot}02$ and $E_b = 1{\cdot}25$, and for the product of the interaction of retinene and protein from retinas (p. 12) $E_a = 1{\cdot}0$–$1{\cdot}05$ and $E_b = 1{\cdot}25$–$1{\cdot}43$ when $E_r = 1{\cdot}00$. Finally, ϵ_r in aqueous digitonin solution $= 39{,}200$. Substituting these numerical values in the relevant equations (1) to (4): $\epsilon_a = 40{,}000n$; $\epsilon_v = 49{,}300p$; $\epsilon_b = 49{,}000m$ and $\epsilon_v = 47{,}200p$. The mean value for ϵ_v will be about $48{,}000p$.

As indicated previously there is good reason to believe that $m = n = 2$. Hence $\epsilon_a = 80{,}000$ and $\epsilon_b = 98{,}000$. At present it is not possible to determine p.

From the result of Schneider *et al.* (1939), $\epsilon_v . \gamma$ (γ = quantum efficiency) = 24,000. Therefore

$$48{,}000p . \gamma = 24{,}000 \quad \text{or} \quad p . \gamma = 0{\cdot}5.$$

If $p = 1$, $\gamma = 0{\cdot}5$; if $p = 2$, $\gamma = 0{\cdot}25$, etc. Therefore γ is at most 0·5 and may be 0·25 or even less.

Retinene reductase (vitamin A dehydrogenase). There is no doubt that crystalline retinene (prepared from vitamin A, Ball, Goodwin & Morton, 1948) is converted by an enzyme present in the retina to vitamin A. However, before the present work was completed, Wald (1949) showed that the process can be realized *in vitro* by means of coenzyme I, retinene reductase and a suitable substrate such as fructose 1:6-diphosphate. Bliss (1949) has shown that this process is reversible.

Conclusions

The structure of indicator yellow is of vital importance to the structure of rhodopsin. It is clear that although transient orange (Lythgoe & Quilliam, 1938) is the primary product of irradiation, indicator yellow is the first reasonably stable product. The original attachment between carrier protein and chromophoric groups is clearly unbroken and the C-N link of the indicator yellow must also occur in rhodopsin.

The nature of this link is probably the same in the indicator yellow analogue as in indicator yellow itself and by implication, the structure of the chromophore is that suggested on p. 16.

Rigid proof is lacking because the analogues have not yet been obtained crystalline and in any case are very readily hydrolysed.

The broad picture of retinal pigments will remain incomplete, however, until a photosensitive substance showing $\lambda_{max.}$ 500 mμ. can be prepared.

SUMMARY

1. The absorption maximum ($\lambda_{max.}$) of alkaline indicator yellow is 365 mμ. and that of acid indicator yellow is 440 mμ.

2. Retinene is present in varying amounts in bleached rhodopsin solutions and appears to be formed by the hydrolysis of indicator yellow.

3. Structures are proposed for indicator yellow.

4. Under certain conditions acid indicator yellow appears to be partially soluble in ethanol.

5. Relationships between the molecular extinction coefficients of rhodopsin, indicator yellow and retinene are discussed.

6. The relationship of indicator yellow to rhodopsin is discussed.

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Studies in Rhodopsin

3. RHODOPSIN AND TRANSIENT ORANGE

BY F. D. COLLINS AND R. A. MORTON
Department of Biochemistry, University of Liverpool

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The existence of transient orange was first established by Lythgoe & Quilliam (1938), who showed that if a rhodopsin solution was irradiated at a temperature near 0°, a comparatively stable orange colour resulted, but if the solution was warmed to room temperature it became much paler (colourless at alkaline pH). The unstable orange substance showed $\lambda_{max.}$ at about 470 mμ. and, on warming, this 'transient orange' changed to 'indicator yellow'. Further work (Broda & Goodeve, 1941) showed that at $-70°$ transient orange was stable and insensitive to light. Lythgoe accordingly formulated the photodecomposition of rhodopsin as follows:

$$\text{rhodopsin} + h\nu \rightarrow \text{transient orange},$$

followed by a thermal process

$$\text{transient orange} \rightarrow \text{indicator yellow}.$$

Lythgoe (1940), in a review of dark adaptation, referred to a possible regeneration of rhodopsin from transient orange, but he did not live to develop his ideas. He also suggested that the regenerated rhodopsin had a maximum differing a little from that of untreated rhodopsin, but did not indicate in which direction the shift occurred.

Chase & Smith (1939) exposed rhodopsin solutions to light of various wavelengths, and with blue light obtained 15% regeneration. The regenerated rhodopsin showed $\lambda_{max.}$ slightly less than 500 mμ., but they were not quite certain that the difference was outside experimental error.

The whole problem seems ripe for reinvestigation.

EXPERIMENTAL

Solutions

Sucrose. 45 g./100 ml. water.

Alum. ($K_2SO_4.Al_2(SO_4)_3.24H_2O$): 4 g./100 ml. water.

Digitonin. A 1% (w/v) solution in water was prepared just before use by boiling the requisite amounts of digitonin and water until all the digitonin had dissolved, and then cooling.

Buffer solutions. McIlvaine's standard buffer solutions were used (Clark, 1928) for the pH range 6–8 and for pH 9·2 a solution of borax ($Na_2B_4O_7.10H_2O$), 19 g./l., was used.

Light petroleum. B.p. 40–60°.

Solid carbon dioxide was used to freeze the solutions, generally in an ethanol bath, which was cooled by the refrigerant to $-70°$.

The absorption spectra were determined by means of the Beckman quartz photoelectric spectrophotometer using a 1 cm. cell.

Preparation of rhodopsin solutions

The procedure was that described by Collins & Morton (1950*a*) and will only be briefly referred to here. All work was done in red light unless otherwise stated.

The retinas from frogs (*Rana temporaria*), rats or bullocks (eyes obtained from an abattoir soon after killing) were shaken with sucrose solution and centrifuged. The supernatant liquid, containing a suspension of rods, was decanted. The suspension was then diluted with water and the rods thrown down by recentrifuging. The rods were then hardened in alum for 2 hr. The alum solution was removed by centrifuging and the rod preparation was then extracted with digitonin solution for 1 hr. The digitonin extract, after centrifuging to remove retinal debris, was then mixed with

an appropriate buffer solution, recentrifuged to ensure optical clarity and was then ready for use.

This procedure was occasionally varied; sometimes the treatment with alum was omitted, or the whole retina was hardened in alum and subsequently extracted with digitonin solution.

The 'regeneration' of rhodopsin

The solution of rhodopsin, obtained as described above, was transferred to a squat 30 ml. beaker. The 2–3 ml. of solution formed a thin layer and were frozen by lowering the base of the beaker into ethanol cooled to $-70°$. After the solution had been kept frozen for 5–6 min. it was illuminated for about 30 min. In the earlier experiments a 100 W. lamp was suspended 30 cm. above the bath containing the frozen rhodopsin solution. In the later experiments a 500 c.p. 'Point-o-lite' lamp was used with a reflector above it and a filter beneath. The filter consisted of a solution of $CuCl_2$ (approx. 10% w/v) which transmitted freely light of wavelengths between 400 and 550 mμ. and effectively cut off infrared radiation.

The freshly frozen rhodopsin solution was pink in colour, but on exposure to light changed to orange. The colour always seemed rather pale, probably because of scattering by the ice crystals. The frozen solution was rotated at intervals to ensure uniform illumination and was also held upside down to make sure that all the rhodopsin was reached by the light.

After irradiation the solution was thawed and kept at room temperature, in complete darkness for 1 hr. The absorption spectrum was then determined; the cells being filled in the dark room (red light) and carried to the Beckman spectrophotometer under a black cloth and placed in position. The instrument has a light-proof compartment for the cells and the intensity of the monochromatic light passing through the cells during the determinations is very small.

The process of irradiating at $-70°$ and then measuring the absorption spectrum at room temperature was repeated several times on the same solution. In one case the absorption spectrum was only measured at some of the stages. For every preparation the final irradiation was carried out at room temperature.

Solutions of pH values 6·7, 7·4 and 7·6 were tried, but the more exact experiments were done at pH 9·2, because of the convenient fact that alkaline indicator yellow has little or no absorption at 500 mμ.

Two experiments were carried out in a different way; in each case 10 ml. of an ox-rhodopsin solution (approx. pH 9) was used, and a 2 ml. sample was taken and its absorption curve measured before and after complete bleaching at room temperature. The remaining 8 ml. were then frozen to $-70°$ and irradiated. After standing at room temperature for 1 hr. in the dark another 2 ml. sample was taken and its absorption measured; it was then fully bleached at room temperature and the absorption measured again. The remaining 6 ml. were again frozen, irradiated at low temperature and the sequence of operations repeated.

RESULTS

Table 1 gives the numerical results obtained in the repeated 'regeneration' of rhodopsin, and displays the main findings. Figs. 1 and 2 illustrate a point not shown in Table 1, namely that the regenerated curves apparently possess an isosbestic point through which the original curve does not pass (Fig. 1), and in the case of rat rhodopsin there appears to be superimposed and changing irrelevant absorption. This makes it necessary to tabulate the findings on selective absorption over a fairly wide range of wavelengths and to note how a constant instrumental limitation will affect the evidence (which will be discussed in detail) for 50% regeneration.

DISCUSSION

A relation between absorption curves for repeated 'regeneration' of visual pigment can be obtained as follows.

Let ϵ = molecular extinction coefficient of rhodopsin at any wavelength λ in the near ultraviolet or visible regions. Also, let c (g. mol./l.) = the initial concentration of the rhodopsin, and after successive regenerations let the concentration of rhodopsin be $r_1c, r_1r_2c, r_1r_2r_3c \ldots, r_1r_2 \ldots r_nc$.

Let a be the extinction of indicator yellow (at wavelength λ) due to the completely bleached rhodopsin expressed as a fraction of ϵ (rhodopsin).

Finally, let the irrelevant absorption at λ be d and the length of the cell be 1 cm.

Therefore the initial extinction

$$= E_0 = c\epsilon + d,$$

after one 'regeneration'

$$= E_1 = r_1c\epsilon + (1 - r_1)\, ac\epsilon + d,$$

after n 'regenerations'

$$= E_n = r_1r_2 \ldots r_nc\epsilon + (1 - r_1r_2 \ldots r_n)\, ac\epsilon + d,$$

after complete bleaching

$$= E_f = ac\epsilon + d.$$

Subtracting the final value from each of the other values we obtain the series:

0	$c\epsilon(1-a)$
1	$r_1c\epsilon(1-a)$
2	$r_1r_2c\epsilon(1-a)$
⋮	⋮
n	$r_1r_2 \ldots r_nc\epsilon(1-a)$.

Hence the ratio of any two successive differences is r_n, and this is independent of c, ϵ or a provided d is constant. If ϵ for the 'regenerated' rhodopsin is different from that of the original rhodopsin a factor for this will be included in r_n. The observed value of r_n will differ from its true value if d (the irrelevant absorption) is not constant during the experiment.

The error in the experimental value of

$$r_n \left(= \frac{E_{n-1} - E_f}{E_n - E_f} \right)$$

can be calculated as follows: if δ is the error (fiducial limits) in E_n, then the error in $(E_{n-1} - E_f)$ will be $\sqrt{(\delta^2 + \delta^2)} = \delta\sqrt{2}$

The error in the quotient $\dfrac{E_{n-1} - E_f}{E_n - E_f}$ will be

$$\frac{E_{n-1} - E_f}{E_n - E_f} \left[\left(\frac{\delta\sqrt{2}}{E_n - E_f} \right)^2 + \left(\frac{\delta\sqrt{2}}{E_{n-1} - E_f} \right)^2 \right]^{\frac{1}{2}},$$

Table 1. *Details of experiments on the regeneration of rhodopsin*

(Solutions prepared from alum-hardened rods, $E_{500\,m\mu}$ measured initially and $\lambda_{max.}$ and $E_{max.}$ measured after irradiation at −70°, followed by 1 hr. at room temperature (1st step); irradiation and regeneration repeated (2nd step) *et seq.*)

Species	Frog					Rat	Ox		Comments
Number of retinas	9	12	12	20	30	60	24	24	
Vol. of rhodopsin solution (ml.)	2·5	2·5	2·5	2·5	2·5	2·5	10*	10*	—
pH	7·6	6·7	8·0	9·2	9·2	9·2	8·80†	9·2	—
Irrelevant absorption:									
Initial $E_{700\,m\mu}$	−0·002	0·013	0·017	0·003	0·003	0·003	0·083	0·014	—
Final $E_{700\,m\mu}$	0·007	0·031	0·068	0·011	0·008	0·006	0·069	0·006	After final bleaching
Final: $E_{500\,m\mu}$	0·016	0·065	0·108	0·027	0·023	0·022	N.M.	N.M.	—
$E_{490\,m\mu}$	0·017	0·071	0·116	0·028	0·025	0·026	N.M.	N.M.	—
Initial $E_{500\,m\mu}$ − final $E_{500\,m\mu}$	0·067	0·158	0·134	0·305	0·293	0·343	0·468	0·789	This measures initial rhodopsin
$E'_{490\,m\mu}$ after first regeneration − final $E_{490\,m\mu}$	0·034	0·085	0·091	N.M.	0·158	0·156	0·263	0·412	This measures initial regeneration of *iso*-rhodopsin
$E''_{490\,m\mu}$ − final $E_{490\,m\mu}$	N.M.	0·046	0·056	N.M.	0·082	0·071	0·145	0·240	Second regeneration
$E'''_{490\,m\mu}$ − final $E_{490\,m\mu}$	N.M.	0·031	0·034	0·045	0·043	0·030	N.M.	N.M.	Third regeneration
$E''''_{490\,m\mu}$ − final $E_{490\,m\mu}$	N.M.	N.M.	N.M.	0·026	0·030	0·016	N.M.	N.M.	Fourth regeneration
$\lambda_{max.}$ mμ. rhodopsin	Approx. 500	500–505	500–505	502	503	497	500	500	—
$\lambda_{max.}$ mμ. *iso*rhodopsin (First regeneration)	Approx. 495	495–500	495–500	495–500	493	487	488	488	—

* From the 10 ml. of extract, a 2 ml. portion was used for measuring the initial $E_{500\,m\mu}$; it was bleached at room temperature and $E_{500\,m\mu}$ remeasured. The remaining 8 ml. were irradiated at −70° and brought to room temperature in the dark and the absorption measured on a 2 ml. portion before and after complete bleaching at room temperature. The remaining 6 ml. was treated similarly, then 4 ml.

† pH meter; all other values nominal (buffer solutions).

N.M. means not measured.

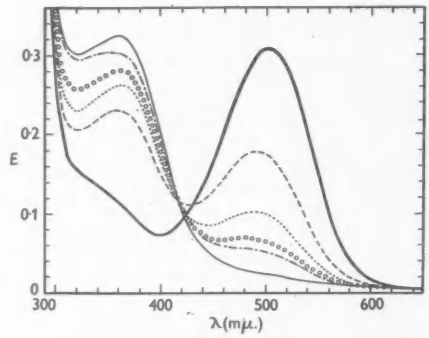


Fig. 1.

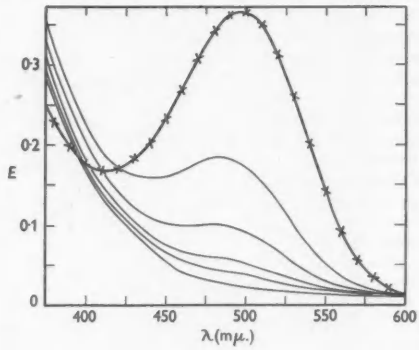


Fig. 2.

Fig. 1. Heavy curve represents fresh unbleached frog rhodopsin solution. This solution was then 'irradiated' at −70° and allowed to reach equilibrium in the dark at room temperature (- - - -). The succeeding curves show the effect of repeating this process three times. The final curve (—light) was obtained after complete bleaching at room temperature. Note that the $\lambda_{max.}$ for rhodopsin differs from that for the regenerated product and that the initial curve does not pass through the approximately isosbestic point.

Fig. 2. Regeneration of rat rhodopsin (cf. Fig. 1). The displacements at shorter wavelengths seem to be due to an increase in irrelevant absorption. Note the displacement of $\lambda_{max.}$.

which is the root square mean of the coefficients of variation. This expression is simplified to

$$\frac{\delta\sqrt{2}}{E_n - E_f}\left[1+\left(\frac{E_{n-1}-E_f}{E_n-E_f}\right)^2\right]^{\frac{1}{2}}$$

or

$$\frac{\delta\sqrt{2}}{E'_\lambda}\left[1+\left(\frac{E''_\lambda}{E'_\lambda}\right)^2\right]^{\frac{1}{2}},$$

if one puts $E_{n-1}-E_f=E''_\lambda$ and $E_n-E_f=E'_\lambda$. The value of δ is usually $\pm 0{\cdot}002$.

Variation in the irrelevant absorption

Solutions of rhodopsin increase in turbidity on standing, as does aqueous digitonin itself. The value of the extinction at 700 mμ. should be almost entirely a measure of turbidity, as rhodopsin does not absorb appreciably in that region. It has been noticed that the value of the extinction at 700 mμ. decreases in some experiments during the 10 or 15 min. required to bleach a rhodopsin solution completely. This, however, is only detectable when the turbidity is appreciable.

How, then, will these disturbances affect the 'regeneration' experiments? Presumably while the solution is frozen the 'turbidity' will be unaffected, but after thawing and during the 1 hr. wait for the solution to reach equilibrium the turbidity may change. As the cause is unknown it is not possible to allow for the effect during an experiment, but sufficiently large changes in the irrelevant absorption could alter the ratio to appreciably more or less than its true value.

Absorption maximum of regenerated rhodopsin

In a previous paper (Collins & Morton, 1950*a*) it was shown that the exact position of the apparent $\lambda_{max.}$ depends on the slope of the irrelevant absorption, but this has to be increased considerably to shift the position of $\lambda_{max.}$ by more than 2 or 3 mμ. Now the changes in the irrelevant absorption which may affect r_n considerably will only have a small effect on $\lambda_{max.}$—perhaps less than 2 mμ. As a result of the first regeneration the maximum of rhodopsin appears to have shifted 5–10 mμ. towards shorter wavelengths. Figs. 1 and 2 illustrate this. This shift always occurs, and varies but little in magnitude. It is also apparent in the difference curves.

Now, if an absorption curve is reduced in intensity (as, for example, by a lowered concentration), but is unchanged in $\lambda_{max.}$, then the ratio of the reduced absorption to the original absorption should be the same at all wavelengths. If, however, the absorption has not only been reduced but also displaced on the wavelength axis, then the ratio will vary at different wavelengths.

In Table 2 the values for r_n at various wavelengths are shown for two experiments. Wavelengths below 420 mμ. are not considered here as the change on bleaching is very small. As will be seen from Table 2, r_1 varies appreciably with wavelength—it increases continuously towards shorter wavelengths—but r_2, r_3, etc., are approximately constant between 430 and 560 mμ. Therefore it may be concluded that after the first 'regeneration' the $\lambda_{max.}$ of rhodopsin has been

Table 2. *'Regeneration' of rhodopsin expressed as a ratio r using absorptions at different wavelengths*

($r_1=E'_\lambda/E_\lambda$, $r_2=E''_\lambda/E'_\lambda$, etc. The estimated experimental errors reflect the increasing importance of instrumental limitations as E values decrease. Error $=\frac{\pm\delta\sqrt{2}}{E_\lambda}\left[1+\left(\frac{E'_\lambda}{E_\lambda}\right)^2\right]^{\frac{1}{2}}$, where $\delta=0{\cdot}002$ and is the error in reading the extinction.)

Frog rhodopsin

λ (mμ.) ...	430	440	450	460	470	480	490	500	510	520	530	540	550	560
r_1	1·00	0·78	0·71	0·67	0·63	0·61	0·57	0·54	0·57	0·51	0·44	0·42	0·39	0·38
Error±	0·09	0·04	0·03	0·02	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·02	0·02	0·03
r_2	0·55	0·53	0·52	0·52	0·52	0·52	0·52	0·52	0·52	0·48	0·50	0·48	0·56	0·49
Error±	0·07	0·05	0·03	0·03	0·02	0·02	0·02	0·02	0·02	0·02	0·03	0·04	0·06	0·09
r_3	0·38	0·45	0·46	0·50	0·51	0·53	0·52	0·51	0·51	0·52	0·52	0·50	0·39	0·41
Error±	0·13	0·09	0·06	0·05	0·04	0·04	0·04	0·04	0·04	0·05	0·06	0·08	0·10	0·18
r_4	1·3	0·87	0·78	0·77	0·69	0·69	0·69	0·69	0·67	0·70	0·70	0·79	0·83	1·0
Error±	0·47	0·24	0·16	0·12	0·10	0·08	0·08	0·08	0·09	0·10	0·13	0·19	0·31	0·57

Rat rhodopsin

r_1	0·78	0·67	0·60	0·56	0·52	0·50	0·46	0·44	0·40	0·37	0·34	0·30	0·30	0·27
Error±	0·04	0·03	0·02	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·02	0·02	0·03
r_2	0·48	0·46	0·46	0·46	0·46	0·45	0·46	0·44	0·45	0·45	0·43	0·41	0·37	0·46
Error±	0·04	0·03	0·02	0·02	0·02	0·02	0·02	0·02	0·02	0·03	0·04	0·05	0·08	0·14
r_3	0·46	0·49	0·46	0·45	0·42	0·45	0·42	0·46	0·41	0·41	0·42	0·35	0·43	0·50
Error±	0·90	0·08	0·06	0·05	0·05	0·04	0·04	0·05	0·05	0·06	0·08	0·13	0·22	0·32
r_4	0·44	0·50	0·48	0·46	0·48	0·48	0·53	0·50	0·50	0·50	0·60	0·50	0·67	0·20
Error±	0·20	0·16	0·14	0·12	0·12	0·10	0·11	0·11	0·13	0·16	0·17	0·40	0·57	0·58

shifted, but that subsequent 'regenerations' have no further effect on its position. Because of these differences it is proposed to call the 'regenerated' material *iso*rhodopsin.

Is the rhodopsin absorption halved?

Table 2 also shows that r_2, r_3 and r_4 are approximately constant and are not greatly different from 0·5. Fig. 1 shows the experiment in which the purest solution of rhodopsin yet obtained was used (Collins & Morton, 1950*a*). Even with this pure solution the increase in turbidity was large, but in the region >430 mμ. it was less than in any other experiment and r_2 and r_3 cannot be far from the true value. It should be noted that the fifth curve (Fig. 1) is slightly anomalous; this is supported by the fact that the irrelevant absorption at 700 mμ. was 0·010 as compared to 0·007 for the curves before and after it.

A precise value for r_n could be obtained with pure stable rhodopsin solutions, but it is already clear that r_n $(n>1)$ is not far from 0·5, and that divergences from 0·5 are consistent with changes in the irrelevant absorption.

It is evident from Fig. 1 that the indicator yellow absorption increases *pari passu* with the decrease in that of rhodopsin and *iso*rhodopsin, but a strictly quantitative treatment of the problem must be deferred at this stage.

Is there an isosbestic point?

If it is true that there are two forms of rhodopsin and only one form of indicator yellow (provided the pH is constant), then after the first regeneration the remaining curves should all pass through an isosbestic point where the molecular extinction coefficient of the indicator yellow is equal to that of the *iso*rhodopsin. However, if the irrelevant absorption should change, an isosbestic point may not be apparent or may be displaced.

In Fig. 1, for example, there appears to be an isosbestic point at about 415 mμ. which the unbleached curve does not pass through. However, it can be shown by considering the slopes of the curves that, if the rhodopsin and *iso*rhodopsin curves are similar in shape, but differ only in $\lambda_{max.}$, the difference between their crossover points with indicator yellow must be less than the interval between their respective maxima. In fact the observed difference is about the same, and this must be due either to the changes in the irrelevant absorption or to differences in the shape of the rhodopsin and *iso*rhodopsin curves.

If the absorption at any wavelength is expressed as a fraction of that at $\lambda_{max.}$ we obtain the results, set out in Table 3, using the data of Table 1 for the difference spectra of ox rhodopsin. These results show that the two curves have exactly the same shape.*

Table 3. *Absorption of rhodopsin and* iso*rhodopsin expressed as a fraction of the maximum absorption*

($\lambda_{max.}$ for rhodopsin 500 mμ., for *iso*rhodopsin 492 mμ.)

$\lambda_{max.}-\lambda_{(m\mu.)}$	−60	−50	−40	−30	−20	−10	0	10	20	30	40	50	60
Rhodopsin	0·39	0·49	0·63	0·77	0·88	0·96	1·00	0·96	0·88	0·74	0·57	0·40	0·26
*iso*Rhodopsin	0·29	0·46	0·61	0·76	0·88	0·97	1·00	0·97	0·89	0·75	0·59	0·41	0·28

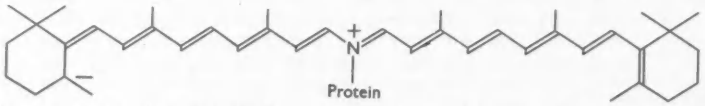
Towards shorter wavelengths we should expect the small differences shown, because the indicator yellow is unaltered and so should affect the two difference curves slightly differently. The close correspondence shown above supports the first alternative, that the irrelevant absorption has changed enough slightly to displace the isosbestic point from its expected position.

Conclusions

It is proposed to consider three working hypotheses which together fit the observed facts. It is recognized that alternatives may be possible for any one or all of these hypotheses. Although they are immediately serviceable, only future experimentation can decide their validity or otherwise.

Hypothesis (1). The conversion of rhodopsin to indicator yellow is an oxidative process involving the loss of two electrons.

Hypothesis (2). The following structure for the rhodopsin chromophoric group is proposed.



(and at least eleven other resonance forms, those with the negative charge on the carbon atoms adjacent to the nitrogen atom being the most probable).

This hypothesis depends partly on hypothesis (1) and partly on the structure proposed previously (Collins & Morton, 1950*b*) for indicator yellow.

Hypothesis (3). The absorption spectrum of *iso*rhodopsin is due to a single rhodopsin chromophoric group on the protein, while that of rhodopsin is due

* $\lambda_{max.}$ rhodopsin, 500 mμ. $\lambda_{max.}$ *iso*rhodopsin, 492 mμ.

to two chromophoric groups adjacently situated on the same protein molecule and interacting to some extent. This hypothesis is independent of the other two.

It is now proposed to examine the experimental results in relation to these hypotheses. The decomposition of rhodopsin can be written as follows:

Rhodopsin $+ nh\nu \rightarrow$ transient orange.

Two transient orange $\rightarrow$ indicator yellow $+$ *iso*-rhodopsin.

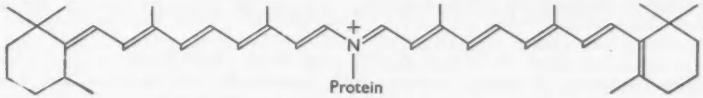
*iso*Rhodopsin $+ nh\nu \rightarrow$ transient orange.

Two transient orange $\rightarrow$ etc.

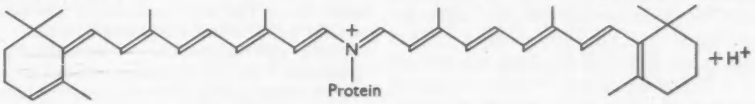
Freezing to $-70°$ stops the reaction at the transient orange stage, while warming to room temperature isolates the thermal reaction. The simplest explanation of this reaction is that transient orange is a free radical, and dismutates as shown above. This implies that rhodopsin loses an electron as follows:

$$\text{Rhodopsin} + nh\nu \rightarrow \text{transient orange} + e^-.$$

Accordingly, the chromophoric group of transient orange can be written:



Dismutation will result in half the transient orange chromophoric groups losing another electron and forming indicator yellow:



This series of reactions accounts for the apparent regeneration reported earlier in this paper.

In a previous paper (Collins & Morton, 1950*b*) it was shown that the molecular extinction coefficient for rhodopsin was about $48{,}000p$ where the concentration was $6{\cdot}06 \times 10^{23}$ chromophoric groups per litre and where p was equal to the number of vitamin A (or retinene) residues per chromophoric group. According to hypothesis (2), p will be equal to 2 and hence $\epsilon_{max.}$ = approx. 96,000.

Schneider, Goodeve & Lythgoe (1939) showed that $\epsilon_{max.} \cdot \gamma$ (= quantum efficiency) = approx. 24,000, and hence γ will be about 0·25 (the overall quantum efficiency). As the dismutation of transient orange reduces the quantum yield by one-half, the quantum efficiency of the primary reaction must be about 0·5. The exact mechanism and energy relationships involved must be left for future work to elucidate, but it is unlikely that one chromophoric group absorbs two quanta. It is more likely that there is either an internal deactivation, or a transference of energy between two activated rhodopsin chromophoric groups.

If the value of $\epsilon_{max.}$ for rhodopsin is about 96,000, and if the results of Broda, Goodeve & Lythgoe (1940) for a minimum value for $E^{1\%}_{1\,cm.}$ (500 mμ.) = 6·6 are accepted, then it follows that the maximum value for the carrier weight is 145,000. The value for the molecular weight is 270,000 (Hecht & Pickels, 1938), and hence there must be two chromophoric groups per molecule.

If these results are compared with the calculations of Collins & Morton (1949) two further results can be deduced. It was shown that $\epsilon_{max.} = 65{,}000\, f$, where f* is the oscillator strength of the absorption band ($\lambda_{max.}$ = 500 mμ.) of rhodopsin. Hence, if $\epsilon_{max.}$ = 96,000, f will = 1·48. It also follows, assuming the molecular weight of 270,000 is correct (and this needs to be confirmed with pure rhodopsin), that $E^{1\%}_{1\,cm.}$ will = 7·1. This value is quite close to that found by Broda *et al.* (1940).

Ordinary rhodopsin is therefore assumed to consist of two chromophoric groups attached to the same protein molecule and manifesting some mutual interaction, thus shifting the $\lambda_{max.}$ slightly towards longer wavelength. As the result of the absorption of light and the subsequent intramolecular dismutation of transient orange, one chromophoric group will have been converted to indicator yellow leaving only one chromophoric group per molecule. This is *iso*rhodopsin, and it will have its $\lambda_{max.}$ at a wavelength characteristic of the isolated chromophoric group. Subsequent dismutation of any transient orange will be intermolecular and will result in the continued formation of *iso*rhodopsin.

Although there are still many gaps in our knowledge of rhodopsin, it is believed that the present theory consolidates the known facts and will act as a guide to future research even if it does not turn out to be correct in all particulars.

SUMMARY

1. If rhodopsin is irradiated at $-70°$ and then allowed to reach equilibrium in the dark at room temperature two phenomena are apparent: (*a*) there

* The oscillator strength is defined as follows:

$$f = 4{\cdot}31 \times 10^{-9} \int \epsilon\, dv,$$

where ϵ = molecular extinction coefficient and v = wave number in cm.$^{-1}$. The limits of the integration are to include an absorption band due to one electronic transition (cf. Collins & Morton, 1949).

is apparently 50% regeneration; (*b*) $\lambda_{max.}$ is shifted about 8 mμ. towards shorter wavelengths.

2. This result has been obtained with frog, rat and ox rhodopsin, and the amount of regeneration is, within the experimental error, 50%.

3. A working hypothesis concerning the structure of the rhodopsin chromophore and the mechanism of its photodecomposition is presented.

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Effect of Sulphonamides on the Aneurin Economy in Animals

By C. C. KRATZING* and E. C. SLATER,†
Australian Institute of Anatomy, Commonwealth Department of Health, Canberra, Australia

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It is well established that many of the toxic effects of sulphonamides on animals fed purified diets are caused by inhibition of bacterial synthesis of some of the B vitamins in the intestine, resulting in a definite syndrome due to the deficiency of these vitamins. Few attempts have been made, however, to determine if the sulphonamides have a more direct effect on the metabolism of the B vitamins in the animal body. In a preliminary report (Slater, 1946), it was shown that sulphadiazine caused an alteration of the aneurin economy in the animal (human and rat), and that this effect was apparently unrelated to bacterial synthesis. Further studies of this effect have now been made, with the primary object of determining whether it possessed any similarity to the action of sulphonamides on micro-organisms, e.g. whether it was reversed by *p*-aminobenzoic acid (PAB).

METHODS

Animals and cages. Hooded rats of an inbred strain were used. They were weaned at 3 weeks and were usually 4–5 weeks old at the start of the experiment. Different groups were carefully matched with regard to litter, sex and body weight. The rats were housed in individual cages with wire-mesh floors. The food consumption was measured to the nearest 0·05 g. Water was provided *ad lib.*

Diets. One of the aims of this investigation was to determine if the effect of sulphonamides on animal tissues was reversed by PAB. It was necessary, therefore, to use a diet which did not contain any PAB. At the time when this work was started (1944–5), it was impossible to obtain sufficient pure folic acid and biotin to prepare a purified diet, essentially free from PAB, containing these two vitamins. Thus the basal diet used in the early experiments was deficient, not only in PAB, but also in folic acid and biotin. Rats fed such a diet without any sulphonamide do not develop biotin or folic acid deficiency, since the two vitamins are normally provided in adequate amounts by bacterial synthesis in the intestine. The addition of sulphonamides to the diet, however, prevents the growth of the bacteria responsible for this synthesis and, consequently, precipitates a folic acid and biotin deficiency. Later, when it was shown that PAB did not reverse the effect of sulphadiazine, it was possible to use a basal diet containing both PAB and sufficient dried liver to supply all the folic acid and probably the biotin required by the rat. It was found that 4% dried ox-liver powder in the diet was sufficient to prevent the blood dyscrasia which occurred when sulphonamides were incorporated in the diet lacking the liver powder. Thus two different diets, *A* (deficient in folic acid, biotin and PAB) and *B* (containing these vitamins) were employed in these experiments. The vitamins whose metabolism was being studied were fed daily to each rat by pipette. The other vitamins were incorporated in the food. The composition of the two diets is given in Table 1.

* Present address, Research Laboratory, Maudsley Hospital, London, S.E. 5.
† Present address, Molteno Institute, University of Cambridge.

Table 1. *Composition of basal diets*

	Amount provided by 100 g. (dry wt.)	
	Diet *A*	Diet *B*
Casein (Labco vitamin-free) (g.)	18	18
Sucrose (g.)	73	68
Salt mixture* (g.)	4	4
Dried whole ox liver† (g.)	—	4
Peanut oil (g.)	2·5	3·1
Cod liver oil (g.)	2	2·5
Wheat germ oil (g.)	0·35	0·43
2-Methyl-1:4-naphthoquinone (mg.)	5	6
Nicotinic acid (mg.)	5	2·5
Pyridoxin (mg.)	0·5	0·25
Inositol (mg.)	16	8
Calcium pantothenate (mg.)	4	2
Choline chloride (mg.)	200	100
p-Aminobenzoic acid (mg.)	—	10
Riboflavin (mg.)	—	0·33

* Hubbell, Mendel & Wakeman (1937).

† Two preparations of dried ox liver were used. The first, used in experiments described in Table 4, provided 17 μg. aneurin/100 g. dry wt. of diet; the second, used in the other experiments, provided 12 μg. aneurin/100 g. dry wt. of diet.

The drugs were incorporated in the food. The percentage of drug in the diet is calculated on a dry weight basis. The amount of drug fed was insufficient to cause gross damage to the kidney or anuria due to precipitation of the drug or its acetyl derivative in the tubules of the kidney, except in one particular case which is discussed below.

Conduct of experiments. It is common in experiments of this type to use the 'paired feeding' technique, i.e. to keep the food consumption of the animals with the greater appetite down to that of those with the smallest appetite, in order to separate specific effects from those due to inanition. Except where otherwise stated, a slight variation of this procedure was followed in the present experiments, in order to allow for possible differences in the efficiency of food utilization. The food consumption of each rat was arranged so that rats from the same litter, which comprised one rat from each group, grew at the same rate as the slowest growing member of the litter, when fed *ad lib.*

Aneurin content of livers. Immediately after removal from the animal, the liver was weighed and treated with 35 ml. acetate buffer, pH 4·5, in a Waring blender for 2 min. Takadiastase (0·1 g.) and papain (0·1 g.) were added, and the treatment continued for 1 min. The mixture was transferred to a stoppered bottle, 5 ml. toluene added and the whole incubated at 37° for 18 hr. The resultant digests were poured through glass wool into a 100 ml. standard flask, and the precipitate washed once with 2 ml. 5N-H_2SO_4 and several times with water, the filtrate being finally made up to 100 ml. The aneurin content of this solution remained unchanged after several weeks at 4°.

The aneurin content was determined by the modification of Jansen's (1936) thiochrome method which has been used in this laboratory for several years, but which has not hitherto been described. A detailed description follows, since the specificity of the method is relevant to the subject of this paper. A 20 ml. sample of the extract was pipetted into a 50 ml. glass-stoppered measuring cylinder and 20 ml. into a flask containing 1 ml. of an aneurin solution (5 μg./ml.). After mixing, 20 ml. of the latter were pipetted into a second measuring cylinder. 20 ml. *n*-Butanol (redistilled and when saturated with water) were added to each cylinder, the contents shaken for 2 min. and then the layers allowed to separate. The two aqueous layers were called *X* and *Y* respectively. Four measuring cylinders received the following solutions, in the order shown, mixing well after each addition.

Reagents	Cylinder no. 1	2	3	4
	(ml. reagents used)			
Methanol (redistilled)	4	4	4	4
7·5N-NaOH	2	2	2	2
Water	0·3	—	0·3	—
5% (w/v) $K_3Fe(CN)_6$	—	0·3	—	0·3
Solution *X*	4	4	—	—
Solution *Y*	—	—	4	4
n-Butanol (saturated with water)	20	20	20	20

The ferricyanide was added just before the solution *X* or *Y*. Exactly 1·5 min. was allowed for the reaction before adding the butanol. Immediately after adding the butanol, the mixture was shaken for 2 min. and poured into a separating funnel. After separation (usually light centrifugation was necessary), the aqueous layer was run off. The measuring cylinder was then rinsed with 4 ml. water which was added to the contents of the separating funnel and the mixture shaken for 1 min. After light centrifugation, if necessary, the aqueous layer was run off and the butanol layer cleared with 2 ml. ethanol. The solution was kept in the dark until its fluorescence was measured (within 3 hr. of preparation) in a Klett photoelectric fluorimeter (Slater & Morell, 1948).

From the four readings in the fluorimeter, the aneurin content of the extract was calculated from the expression, aneurin content of extract

$$= \frac{0{\cdot}25(R_2 - R_1)}{1{\cdot}05(R_4 - R_3) - (R_2 - R_1)}\ \mu\text{g./ml.},$$

where R_1, R_2, etc. represent the fluorimeter readings from the four cylinders.

With several different types of materials analysed in this laboratory it has been found essential to use the 'internal standard' procedure, since the conversion of aneurin to thiochrome is not quantitative and may differ considerably in different materials. For the same reason, it is necessary to standardize rigorously the procedure in order to obtain reproducible readings, and duplicate measurements are desirable. Separate 'blank' measurements in the two solutions *X* and *Y* are necessary, since it was found that shaking an alkaline solution of aneurin with air, under the conditions employed in preparing the 'blank' solution, causes the oxidation of about 2·5% of the aneurin to thiochrome.

Allowance was made for the aneurin content of the takadiastase and papain, which was determined separately. In a separate control experiment, a rat liver was halved. A known amount of pure aneurin was added to one half and the aneurin contents of both halves determined as above. The recovery of added aneurin was 92% of the theoretical. The standard error of a single determination by this method was 3·7% of the mean.

The specificity of this method for rat liver was tested on the extract of a liver of a rat fed a very low aneurin diet by destroying the aneurin, both in the extract itself and in the

extract to which aneurin was added, by treatment with 2% (w/v) Na_2SO_3 at pH 5 and 100° for 30 min. (cf. Schultz, Atkin, Frey & Williams, 1941; Slater & Rial, 1942). After this treatment, all four readings (R_1–R_4) were about the same. Thus, if there is any substance in the rat-liver extracts which interferes with the aneurin determination by contributing to the value obtained, it must be similar to aneurin in the following respects: (1) not extracted by butanol from an acid aqueous solution; (2) converted by alkaline ferricyanide to a blue-fluorescent compound which is extractable by butanol from alkaline aqueous solution (even if the substance is, itself, blue-fluorescent and is extractable by butanol from alkaline aqueous solution, it will not affect the determination unless the fluorescence is altered by oxidation); (3) any fluorescent compound formed in this way must be activated by light of approximately the same wavelength as for thiochrome and its fluorescence spectrum must be similar (to the eye, the fluorescence of the solution prepared from the liver extract was similar to that of a solution of thiochrome); (4) it must be destroyed by sulphite to give compounds not interfering in the thiochrome test. It is considered that it is very unlikely that the extracts will contain a substance resembling aneurin in all these respects, and that the specificity of the thiochrome method compares favourably with that of most biochemical analyses.

Sulphadiazine was not itself fluorescent when tested in a concentration (1%) much greater than that found *in vivo*, nor did it give a fluorescent compound on oxidation.

Aneurin content of carcass of rat after removal of liver. The rat was killed by decapitation and the blood allowed to drain into 100 ml. acetate buffer, pH 4·5. The carcass was skinned and the liver and kidneys removed and weighed, the liver being treated as above and the kidneys added to the acetate buffer. The gastro-intestinal tract was also removed, flushed out with water and then added to the buffer solution. The remainder of the carcass, including the head, was cut up with scissors, added to the buffer, and the whole treated in a Waring blender for 2 min. Takadiastase (1 g.) and papain (1 g.) were added and the treatment continued for 1 min. The skin and fur were cut up and added to the mixture which was then treated in the blender for a further 2 min. Toluene (20 ml.) was added and the mixture incubated at 37° for 24 hr. H_2SO_4 (20 ml. of 5N) was then added and the mixture filtered through glass wool. The precipitate was washed five times with water and the filtrate allowed to settle in a separating funnel. The aqueous layer was separated from the toluene layer and diluted to 500 ml. The aneurin content of the extract was determined as above. The sulphite procedure on both control and sulphadiazine-fed rats showed that the thiochrome method was specific when applied to the rat carcasses.

Riboflavin contents of the same extracts were determined by the method of Slater & Morell (1946).

Pyruvate content of blood. In order to obtain the 'resting value' of the pyruvate in the blood, the animal's movements were restricted for some hours before killing by placing it in a closely fitting tube with wire mesh at the ends. For the same reason, the animals were usually fasted overnight before killing by decapitation. The blood was collected into a weighed 10 ml. stoppered centrifuge tube containing 4 ml. of 0·7N ice-cold trichloroacetic acid. The tube was shaken, reweighed, allowed to stand 20 min. at 0° and centrifuged. A 2 ml. sample of the supernatant was used for the pyruvate determination and the remainder for sulphadiazine determination. Pyruvate was determined by the 'method A' procedure of Friedemann & Haugen (1943). The colour was measured in a Spekker absorptiometer, using an Ilford 601 filter and the concentration of pyruvate was calculated from a standard curve obtained at the same time with Na pyruvate (98·5% pure), prepared from crude pyruvic acid by the method of Lardy (1945). This method is not specific for pyruvate, but estimates other ketonic acids, e.g. α-ketoglutarate. Sulphadiazine did not give a colour in this reaction.

Sulphadiazine (free + acetylated) was measured in the trichloroacetic acid extract by Fantl's (1940) method.

Statistics. The results were treated by the analysis of variance technique as described by Goulden (1939).

RESULTS

Effect of aneurin intake on aneurin content of liver. For purposes of comparison with the effect of sulphonamides, a preliminary experiment to determine the effect of changes in the aneurin intake of the rats, in the absence of sulphonamides, was carried out, with results shown in Fig. 1. There is an approximately linear relationship between the aneurin content of liver and the dietary intake over the range 7·6–20·7 μg./day. Apparently 20·7 μg./day is not sufficient to saturate the tissues in young rats, since the curve shows no signs of reaching a maximum.

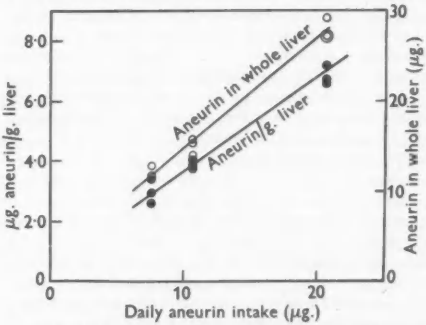

Fig. 1. Effect of different dietary intakes of aneurin on the concentration of aneurin in and the total aneurin content of rat liver. Female rats, 33–35 days old at commencement of experiment, divided into three groups of three rats each, fed diet *B* plus 6·9, 10, 20 μg. aneurin per day, respectively. Diet *B* provided additional 0·7 μg. aneurin per day. Rats killed after 28 days, when still increasing in weight. Each point represents a single rat: •, μg. aneurin/g. liver; ○, μg. aneurin in whole liver. Mean body weights: 43·8 g. at commencement of experiment, 77·6 g. at end.

Effect of sulphadiazine and succinylsulphathiazole on rats fed diet A. It was essential to investigate the possibility that the effect of sulphadiazine on the aneurin metabolism of rats fed diet *A* was an indirect consequence of the folic acid and biotin deficiency which inevitably occurs when sulphonamides are included in such a purified diet. Consequently, the

effect of sulphadiazine, which is readily absorbed from the intestine, was compared with that of succinylsulphathiazole, which is only slightly absorbed. The two drugs would be expected to have essentially the same effect on the intestinal bacteria (the non-absorbable drug might possibly cause a greater bacteriostasis than the absorbable), while only the drug which is absorbed could be expected to have a marked direct effect on the tissues of the animal.

The experiment described in Table 2 was carried out to test the effect of these two drugs on both the aneurin and riboflavin economy of the rat. Consequently, the amounts of aneurin (6·9 μg./day) and riboflavin (10·3 μg./day) given were distinctly less than required for adequate nutrition. Histological examination of the kidneys failed to show any evidence of damage, which is confirmed by the fact that the kidneys of the rats fed sulphadiazine were no heavier than those of the control group. Kornberg, Endicott, Daft & Sebrell (1945) found that kidneys damaged by feeding excessive amounts of the soluble sulphonamides were abnormally heavy.

by sulphadiazine and (2) to determine if the increased aneurin concentration found in the liver was confined to that tissue alone or was also found in other tissues. The rats received the same amount of aneurin as in the first experiment, but the amount of riboflavin was reduced to 6·9 μg./day, in order to determine if the failure to find any effect of sulphadiazine on the riboflavin economy in the first experiment was due to the rat receiving so much riboflavin that its tissues were virtually saturated. The concentrations of riboflavin in the liver in the previous experiment were practically the same as those found by Kratzing (1947) in rats fed 30 μg./day.

The most important conclusion to be drawn from Table 3 is that PAB, in an amount equimolar with the sulphonamide, has no significant effect, either on its own, or in reversing that of sulphadiazine. Sulphadiazine, itself, had essentially the same effect as in the previous experiment. The only difference is that the effect in the weight of the liver and the kidney is not statistically significant in this experiment. This is, however, not inconsistent with the

Table 2. *Effect of sulphadiazine and succinylsulphathiazole on rats fed diet A*

(Twelve female rats, aged 29 days, were divided into three groups, each containing one rat from each of four litters. Group 1 was fed diet *A*; group 2, diet *A* + 0·55 % sulphadiazine; group 3, diet *A* + 0·55 % succinylsulphathiazole. Each rat received orally 6·9 μg. aneurin and 10·3 μg. riboflavin/day. The weight of the rats had reached a plateau by the 23rd day of the experiment and remained practically constant for the following 9 days, after which (on the 32nd day) the animals were killed and the livers analysed.)

	Mean of rats in			Statistical significance of difference between any two group means*	Smallest difference between group means which is statistically significant at level	
	Group 1	Group 2	Group 3		$P = 0·05$	$P = 0·01$
Gain in wt. at end of experiment (g.)	27·2	27·5	27·0	NS	—	—
Liver wt. (% body wt.)	3·84	4·23	3·72	S	0·31	0·46
Kidneys wt. (% body wt.)	1·12	1·00	1·11	S	0·065	0·099
Aneurin content (μg./g. liver)	3·71	5·79	3·54	S	1·01	1·53
Aneurin content (μg./whole liver)	9·8	16·6	8·9	S	2·3	3·6
Riboflavin content (μg./g. liver)	33·1	30·0	32·2	NS	—	—
Riboflavin content (μg./whole liver)	87	86	81	NS	—	—

* S = significant; NS = not significant.

Table 2 shows that sulphadiazine caused the following changes, all of which were statistically significant: (1) the weight of the liver was increased by 10 %; (2) the weight of the kidneys was decreased by 11 %; (3) the concentration of aneurin in the liver was increased by 56 %, and the total aneurin in the liver by 69 % compared with group 1; the corresponding percentage increases were 64 and 87 % when group 3 was taken as the basis of comparison. On the other hand, succinylsulphathiazole had none of these effects. Neither drug affected the riboflavin content of the liver.

Effect of PAB, both alone and in conjunction with sulphadiazine, on rats fed diet A: The experiment described in Table 3 had two objects in view, (1) to determine if PAB had any effect on the upset caused

findings in the first experiment, but is due to the fact that the variation between individual animals of the same sex in the same litter was much greater in this experiment than in the first, when more animals were available so that more careful matching of the animals with respect to initial body weight was possible. Subsequent experiments (see p. 29) confirmed the effect of sulphadiazine on the weight of the liver and kidney found in the previous experiment. The analyses of the rat minus the liver show that the effect of sulphadiazine on the tissues is not confined to the liver. The concentration of aneurin in the whole rat less its liver was 33 % greater in rats fed the drug, compared with that of control rats (combined groups 3 and 4 versus combined groups 1 and 2), and this difference was statistically significant.

Table 3. *Effect of* p-*aminobenzoic acid* (*PAB*), *alone or in conjunction with sulphadiazine, on rats fed diet A*

(Fifteen rats, aged 26–31 days, were arranged in four groups, three groups (no. 1, 2 and 4) containing one rat from each of four litters, while the other group (no. 3) contained one rat from each of three of these litters. The rats from three of the litters were all male, those from the other litter were all female. Group 1 received diet *A*; group 2, diet *A* + 0·3 % PAB (equimolar with 0·55 % sulphadiazine); group 3, diet *A* + 0·55 % sulphadiazine; group 4, diet *A* + 0·55 % sulphadiazine + 0·3 % PAB. Each rat received orally 6·9 μg. aneurin and 6·9 μg. riboflavin/day. Maximum weights were reached between the 15th and 18th day; weights then declined rather rapidly for a few days, but were practically stationary for 2–3 days before the termination of the experiment (23rd and 24th days).)

	Mean of rats in				Statistical significance of difference between any two group means†	Smallest difference between group means which is statistically significant at level	
	Group 1	Group 2	Group 3*	Group 4		$P = 0·05$	$P = 0·01$
Gain in wt. at end of experiment (g.)	17·5	17·9	18·0	17·6	NS	—	—
Liver wt. (% body wt.)	4·15	4·10	4·08	4·30	NS	—	—
Kidneys wt. (% body wt.)	1·23	1·18	1·08	1·12	NS	—	—
Aneurin content (μg./g. liver)	3·21	3·47	5·94	5·24	S	0·81	1·27
Aneurin content (μg./whole liver)	9·2	9·3	16·1	15·5	S	2·0	3·1
Aneurin content (μg./g. whole rat less liver)	0·81	0·82	1·07	1·10	S	0·23	0·37
Aneurin content (μg./whole rat less liver)	47	47	62	64	S	10	16
Riboflavin content (μg./g. liver)	24·9	25·4	27·7	26·2	NS	—	—
Riboflavin content (μg./whole liver)	73	69	77	78	NS	—	—
Riboflavin content (μg./g. whole rat less liver)	3·19	3·14	2·76	2·74	S	0·40	0·61
Riboflavin content (μg./whole rat less liver)	189	181	164	160	S	20	31

* Mean after estimating 'missing' value by technique of Goulden (1939).
† S = significant; NS = not significant.

As in the previous experiment, the drug had no appreciable effect on the riboflavin in the liver, although the values were considerably lower than in the first experiment, so that any effect of sulphadiazine on the riboflavin values similar to that on the aneurin should have been revealed in this experiment. Sulphadiazine had a small but statistically significant effect on the riboflavin content of the rat carcass from which the liver was removed. This effect, which amounted to only 12 %, was in the opposite direction to that on the aneurin content. It may be due to suppression of bacterial synthesis of riboflavin by the drug, although one might expect that PAB would reverse such a suppression and this was not found. It is interesting to note that the riboflavin contents of both the liver and of the whole rat are very much higher than the corresponding values for aneurin, despite the fact that it is generally considered that the riboflavin requirement of animals is about 50 % more than that for aneurin. This is an illustration of the fact already discussed by Ferrebee & Weissman (1943) and Kratzing (1947) that tissue catabolism, caused in this case by the deficiency of aneurin, has a strong sparing action on the rat's requirement for riboflavin.

Effect of sulphadiazine on rats fed diet B. Since it now seemed likely that sulphadiazine had little effect on the riboflavin economy of the rat, an optimal concentration of this vitamin was included in the basal diet, which also included the liver powder to provide adequate amounts of biotin and folic acid. Thus the basal diet was adequate in all the known dietary essentials with the exception of aneurin, 7·9 μg. of which was provided orally per day. The experiment reported in Table 4 showed that the effect of sulphadiazine was the same as in the earlier experiments when the diet was deficient in folic acid and biotin. This result was confirmed in a second experiment in which two groups, each of six young male rats, were fed diet *B*, giving a total aneurin intake of 8·3 μg./day, and the experimental group received 0·33 % sulphadiazine. After 21 days the control animals contained 4·11 μg./g. aneurin in the liver (14·4 μg. aneurin in the whole liver) and those of the experimental group contained 5·16 μg./g. aneurin in the liver (20·6 μg. aneurin in the whole liver). The increase in the aneurin content per gram liver and total aneurin content of liver were statistically significant ($P < 0·001$).

The effect of sulphadiazine on rats receiving much more aneurin (20·7 μg./day) was also studied. The control animals contained 6·84 μg./g. liver (27·9 μg. in the whole liver), while those receiving the drug contained 8·71 μg./g. (37·6 μg. total aneurin). Thus the absolute increase was of the same order as in the experiments with a smaller amount of aneurin.

Comparison of effects of oral and injected sulphadiazine on the aneurin content of the liver and the pyruvate content of the blood, using diet B. Since the metabolism of pyruvate is impaired in aneurin

Table 4. *Effect of sulphadiazine on rats fed diet B*

(Fourteen female rats, aged 23–28 days, divided into two groups, each containing one rat from each of seven litters. Group 1 was fed diet *B*, group 2, diet *B* + 0·55% sulphadiazine. Each rat received orally a supplement of 6·9 μg. aneurin/day. The diet provided 1·0 μg. aneurin/day, so that total intake was 7·9 μg. The rats were killed on the 28th day of the experiment.)

	Mean of rats in		Smallest difference between group means which is statistically significant at level	
	Group 1	Group 2	$P = 0.05$	$P = 0.01$
Gain in wt. at end of experiment (g.)	40·8	40·1	1·0	1·5
Liver wt. (% body wt.)	3·68	4·26	0·62	0·93
Kidneys wt. (% body wt.)	1·07	0·96	0·05	0·08
Aneurin content (μg./g. liver)	4·89	6·05	0·71	1·08
Aneurin content (μg./whole liver)	11·9	16·8	2·4	3·6
Aneurin content (μg./g. rat less liver)	0·35	0·58	0·17	0·26
Aneurin content (μg./whole rat less liver)	22	36	10	14

Table 5. *Comparison of effects of oral and injected sulphadiazine on the aneurin content of the liver and the pyruvate content of the blood, using diet B*

(Exp. 1. Eighteen male rats, 23–31 days old, divided into three groups of six rats each. Group 1 was fed diet *B*; group 2, diet *B* + 0·55% sulphadiazine; group 3, diet *B*. Group 3 rats were injected four times daily with 12·5 mg. of sulphadiazine (as Na salt), i.e. total of 50 mg./day. All rats received a total of 7·6 μg. aneurin/day. Experiment terminated on 28th day. Blood samples for determination of sulphadiazine and pyruvate taken at time of killing.)

	Mean of rats in			Statistical significance of difference between any two group means*	Smallest difference between group means which is statistically significant at level	
	Group 1	Group 2	Group 3		$P = 0.05$	$P = 0.01$
Average daily intake of sulphadiazine (mg.)	0	32·2	50	—	—	—
Gain in wt. at end of experiment (g.)	30·5	28·5	28·7	NS	—	—
Sulphadiazine (mg./100 ml. blood)	—	6·2	8·9	S	2·4	3·8
Liver wt. (% body wt.)	4·12	4·31	4·44	NS	—	—
Kidneys wt. (% body wt.)	1·079	0·986	1·055†	S	0·041	0·059
Aneurin content (μg./g. liver)	3·89	6·79	6·66	S	1·01	1·43
Aneurin content (μg./whole liver)	12·1	21·3	21·7	S	2·49	3·53
Pyruvate (mg./100 ml. blood)	2·40	1·30	0·84	S	0·40	0·56

* S = significant; NS = not significant.

† Omitting one rat in this group which had abnormally heavy kidneys and filling 'missing value' by method of Goulden (1939).

(Exp. 2. Seventeen male rats, aged 35–39 days, divided into three groups, groups 1 and 2 of six rats each; group 3, five rats. Group 1 fed diet *B*; group 2, diet *B* + 0·55% sulphadiazine; group 3, diet *B*. Group 3 rats were injected with 15 mg. sulphadiazine (as Na salt) once a day. All rats received a total of 7·85 μg. aneurin/day. Experiment terminated on the 21st day. Blood samples for determination of sulphadiazine and pyruvate taken at time of killing.)

	Mean of rats in			Statistical significance of difference between group means†	Smallest difference between group means which is statistically significant at level	
	Group 1	Group 2	Group 3*		$P = 0.05$	$P = 0.01$
Av. daily intake of sulphadiazine (mg.)	0	44·2	15	—	—	—
Gain in wt. at end of experiment (g.)	27·7	25·4	27·7	S	1·2	1·7
Sulphadiazine (mg./100 ml. blood)	—	11·5	4·3	S	5·0	7·4
Liver wt. (% body wt.)	4·31	5·05	4·51	S	0·25	0·37
Kidneys wt. (% body wt.)	1·007	0·961	1·092	S	0·068	0·112
Aneurin content (μg./g. liver)	3·68	5·19	4·35	S	1·02	1·46
Aneurin content (μg./whole liver)	13·3	21·0	16·3	S	3·6	5·2
Pyruvate (mg./100 ml. blood)	3·96	2·96	3·20	S‡	0·79‡	1·05‡

* Mean after estimating 'missing' value by technique of Goulden (1939).

† S = significant; NS = not significant.

‡ The standard error used in the calculation of the significance was, in this case, derived from pooling all the blood pyruvate determinations in this and other experiments.

deficiency, pyruvate accumulates in the blood and tissues of aneurin-deficient animals (Thompson & Johnson, 1935). It was therefore of interest to determine whether the increased tissue levels of aneurin resulting from dosage with sulphadiazine were accompanied by a reduced level of pyruvate in the blood. This was one of the objects of the experiments which are summarized in Table 5. The other object was to determine if sulphadiazine had to pass through the alimentary canal before it took effect. The effects of sulphadiazine, given orally and by subcutaneous injection, were compared. In Exp. 1, a total of 50 mg. sulphadiazine per day was injected in four separate portions. This procedure was successful in maintaining a high concentration of the drug in the blood; in fact, the concentration of the drug in the blood was 45% greater in the injected animals than in those receiving the drug orally. In Exp. 2, however, 15 mg. of the drug was administered only once per day, and this was insufficient to maintain an appreciable concentration of the drug. In Exp. 1, the rats were fasted overnight before killing in order to obtain more reliable pyruvate values (Lu, 1939). This probably caused a decrease in the amount of sulphadiazine in the blood.

Table 6. *Effect of sulphadiazine. Summary of figures in Table 5*

Exp. no.	Method of administration of drug	Change from control group of: Blood sulphadiazine (mg./100 ml.)	Blood pyruvate (mg./100 ml.)	Liver aneurin (μg./g.)
1	Oral	+6·2	−1·1	+2·9
	Injected (4 × 12·5 mg./day)	+8·9	−1·6	+2·8
2	Oral	+11·5	−1·0	+1·5
	Injected (1 × 15 mg./day)	+4·3	−0·8	+0·7

The following conclusions may be drawn from Table 5: (1) High concentrations of sulphadiazine in the blood are accompanied by increased tissue levels of aneurin and reduced blood pyruvate. There was a highly significant negative correlation ($r = -0{\cdot}76$; $P < 0{\cdot}001$) between the blood-pyruvate level and the aneurin content of the whole liver in all rats in Exp. 1. (2) The sulphadiazine had essentially the same effect whether it was fed or injected. In Exp. 1 where more sulphadiazine was injected than fed, with the result that the blood sulphadiazine was higher in the injected animals than in those given the drug orally, the blood pyruvate was significantly lower in the former than in the latter. Both groups had approximately the same amount of aneurin in the livers. In Exp. 2, in which considerably less sulphadiazine was injected than fed, which is reflected in the blood sulphadiazine values, the aneurin content of the liver and the pyruvate in the blood of the injected animals were intermediate between the control animals and those fed the drug. The correlation between high blood sulphonamide, high liver aneurin and low blood pyruvate is clearly shown in Table 6, which is calculated from the figures in Table 5. Owing to different experimental conditions, the figures obtained in the different experiments are not directly comparable.

As in previous experiments, the livers of animals given sulphonamide were heavier than those of the controls and this difference is also independent of the method of administration of the drug. The small amount of drug given to group 3 in Exp. 2 produced a small increase in the liver weight, which was nearly statistically significant at the $P = 0{\cdot}05$ level. The kidneys of the injected animals were definitely heavier than those of the rats fed the drug and also than those of the control rats in Exp. 2. This was not found in Exp. 1, except in one rat of group 3, in which the kidneys were about twice as heavy (expressed as percentage of body weight) as those of the other rats. This animal grew much more slowly than the others after the first 2 weeks of the experiment, so that during the last 2 weeks, it was receiving considerably more sulphonamide per unit body weight than the other rats in this group. Its blood sulphadiazine was the highest in the experiment. The greater kidney weight is very probably due to kidney damage caused by precipitation of the drug in the kidney tubules (Kornberg *et al.* 1945). The liver aneurin content of this rat was little different from that of other rats given sulphadiazine, which indicates that the toxic action of sulphadiazine caused by kidney damage is not connected with the effect on the tissue aneurin levels. This rat, which was receiving the most sulphadiazine, also had the heaviest liver (5·0% body weight compared with 4·12% in the control group).

Effect of different sulphonamides and 2-amino-4-methylpyrimidine. In Table 7, the effects of sulphadiazine are compared with those of equimolecular amounts of other sulphonamides, viz. sulphanilamide, sulphapyridine, sulphathiazole and sulphamerazine. The following conclusions may be drawn: (1) Sulphamerazine, like sulphadiazine, caused a considerable increase in the aneurin content of the liver and a decrease in the blood pyruvate and kidney weight. These effects of sulphamerazine

Table 7. *Effect of different sulphonamides*

(Twenty-four male rats, 23–29 days old, divided into six groups of four each, fed diet *B* for 28 days. Total aneurin intake 7·6 μg./day. Group 1, control diet; group 2, diet + 0·38 % sulphanilamide; group 3, diet + 0·55 % sulphapyridine; group 4, diet + 0·55 % sulphadiazine; group 5, diet + 0·56 % sulphathiazole; group 6, diet + 0·58 % sulphamerazine. 'Paired feeding' method used. Blood samples for pyruvate analyses taken at time of killing.)

	Mean of rats in groups						Statistical significance of difference between group means*	Smallest difference between group means which is statistically significant at level	
	1	2	3	4	5	6		$P = 0·05$	$P = 0·01$
Gain in wt. at end of experiment (g.)	31·8	34	29·8	36·0	36·8	31·2	S	4·7	6·5
Liver wt. (% body wt.)	4·16	4·01	4·59	4·11	4·37	4·50	S	0·36	0·50
Kidneys wt. (% body wt.)	1·07	1·09	1·13	0·94	1·03	0·85	S	0·10	0·14
Thyroid wt. (mg.)	10·4	10·0	13·3	29·1	10·0	30·8	S	5·6	7·7
Aneurin content (μg./g. liver)	3·52	3·92	3·74	5·44	3·70	6·25	S	0·72	1·00
Aneurin content (μg./whole liver)	10·9	12·3	12·8	17·9	13·2	21·4	S	2·4	3·4
Pyruvate (mg./100 ml. blood)	2·5	2·1	2·5	1·8	2·6	1·4	S	1·0†	1·3†

* S = significant; NS = not significant.

† The standard error used in the calculation of the significance was, in this case, derived from pooling all the blood-pyruvate determinations in this and in other experiments.

Table 8. *Effect of 2-amino-4-methylpyrimidine on rats*

(Eight male rats, 39 days old, divided into two groups of four each, fed diet *B* for 21 days. Total aneurin intake, 7·8 μg./day. Group 1, control diet; group 2, diet + 0·24 % 2-amino-4-methylpyrimidine. 'Paired feeding' technique.)

	Mean of rats in		Smallest difference between group means which is statistically significant at level	
	Group 1	Group 2	$P = 0·05$	$P = 0·01$
Gain in wt. at end of experiment (g.)	33·6	37·8	3·5	6·5
Liver wt. (% body wt.)	5·75	5·95	0·46	0·84
Kidneys wt. (% body wt.)	1·05	0·98	0·05	0·09
Thyroid (mg.)	5·65	7·3	1·9	3·4
Aneurin content (μg./g. liver)	3·33	3·07	0·74	1·37
Aneurin content (μg./whole liver)	15·9	14·8	5·7	10·4
Pyruvate (mg./100 ml. blood)	1·9	1·8	0·4	0·7

were, in fact, somewhat greater than those of sulphadiazine. (2) Sulphanilamide had no effect, sulphapyridine caused a significant increase in the liver weight, but otherwise had no effect, while the only significant effect of sulphathiazole was on the weight gain (all groups received the same food intake).

In this experiment the thyroids were weighed, since Mackenzie & Mackenzie (1943) had shown that certain sulphonamides caused an increased weight of the thyroid. It is most noticeable that the two drugs which had the effect on the aneurin and pyruvate levels were the only two to increase the thyroid weight and this increase amounted to trebling the weight.

Since the only drugs which had the effect studied in this paper were sulphadiazine and the very closely related sulphamerazine (which differs from sulphadiazine only in having one methyl group substituted in the pyrimidine ring), the effect of the same concentration of the simple pyrimidine, 2-amino-4-methylpyrimidine, was tried in the experiment summarized in Table 8. This compound was, however, without any effect on the aneurin and pyruvate levels. It appeared to have some stimulatory effect on growth and caused a lower kidney weight (expressed as a percentage body weight). The comparison of the kidney weights in this experiment is, however, complicated by the difference in the weight of the animals.

Survival experiments. Two groups of ten male rats (30–36 days old) were fed *ad lib.* a diet the same as diet *B* except that it provided 3 μg. aneurin/10 g. dry weight of food; no additional aneurin was provided. One group received 0·33 % sulphadiazine in the diet. Both groups survived for about the same time, approximately 40 days, but it was noticeable that the typical aneurin-deficiency symptoms were shown only by the rats in the control group. These symptoms were: (1) drop in food consumption, (2) cessation of growth followed by fairly rapid decline in weight, (3) typical hump back, (4) rough fur, (5) the onset of priapism in some, and (6) lack of co-

ordination and convulsions. The rats fed the drug, on the other hand, lost much less weight (only about 10% of the maximum) and did not show these typical signs of aneurin deficiency. They remained in a crouched position for long periods of time, all movements apparently being painful; the fur was in good condition, but felt cold. The animals lived 3–5 days after the onset of these toxic symptoms and died of convulsive seizures.

DISCUSSION

From the above experiments it can be concluded that the inclusion in the diet of 0·55% sulphadiazine or sulphamerazine (but not of equimolar amounts of the other sulphonamides tried) has a marked effect on the aneurin economy of young rats. This is characterized, not only by an increased concentration of aneurin in the tissues of the animals fed the sulphonamide, but also by decreased blood-pyruvate levels. Rats on a very low aneurin diet, when fed sulphadiazine, did not show the typical aneurin-deficiency symptoms seen in controls not given the drug. The magnitude of the effect on the aneurin economy in a typical experiment (Exp. 1, Table 5) can be calculated from the increase in the aneurin content of the liver in this experiment and the relation between aneurin intake and liver content shown in Fig. 1. This calculation showed that supplementing the diet with 0·55% sulphadiazine was equivalent to increasing the aneurin intake from 7·6 to 15·0 μg./day, i.e. to very nearly doubling the aneurin intake.

No systematic study was made of the effect of different amounts of drug or of the time of treatment on the effect on the aneurin economy, but there are indications that 0·55% sulphadiazine in the food is probably nearly sufficient for the maximum effect. Thus, when the amount of sulphonamide given was increased (Exp. 1, Table 5), there was no appreciable increase in the aneurin content of the liver (although the blood-pyruvate level was somewhat lower). On the other hand, when less sulphadiazine was injected (Exp. 2, Table 5) the effect on the aneurin and pyruvate levels was appreciably less.

During the course of this investigation, several possible explanations of this effect were considered, but were eliminated as the result of further experiments. These were: (1) an effect on the renal threshold of aneurin, (2) change of bacterial flora in the intestine with altered vitamin synthesis, (3) a consequence of the extensive acetylation of the sulphonamide, and (4) an inhibition of intracellular enzymes containing aneurin.

(1) *Renal threshold.* If the sulphonamide increased the renal threshold of aneurin, increased aneurin concentrations in the tissues might be expected. However, an increased renal threshold would cause a decrease of the aneurin excretion which is contrary to the finding previously reported (Slater, 1946) that, in man, the urinary excretion of aneurin was greatly raised by ingestion of sulphadiazine. Kinnunen (1946) found that a number of sulphonamides did not affect the aneurin excretion of rats, but this is not inconsistent with the present findings, since Kinnunen did not use either sulphadiazine or sulphamerazine, which were the only two sulphonamides found effective in the present study. In any case, Kinnunen used aneurin-deficient rats in these experiments and a considerable change in the aneurin economy of such rats would not be reflected by an appreciable rise in the urinary excretion of aneurin.

(2) *Bacterial synthesis.* Ellinger and his coworkers (Ellinger & Emmanuelowa, 1946; Ellinger, Abdel Kader & Emmanuelowa, 1947) have shown that the administration of ambamide (*p*-aminomethylbenzenesulphonamide) increased the urinary excretion of *N*-methylnicotinamide by man and rats. This is due to a change of the intestinal flora, brought about by the drug, leading to an increased intestinal synthesis of nicotinamide. It was possible that sulphadiazine had a similar effect on the bacterial synthesis of aneurin. This was, however, considered unlikely for the following reasons: (*a*) The therapeutic sulphonamides suppress rather than increase the intestinal synthesis of the B vitamins. (*b*) The amount of aneurin available to the rat from intestinal synthesis when fed a high sucrose diet of the type used in these experiments must be very small, since rats very soon die of aneurin deficiency, unless the diet is supplemented with aneurin. (*c*) Since succinylsulphathiazole is not absorbed, but remains in the intestine, and has much the same type of effect on bacteria as sulphadiazine, it would be expected, on this hypothesis, that succinylsulphathiazole would have a greater effect on the aneurin levels than sulphadiazine. Actually it was found (Table 2) that succinylsulphathiazole had no effect. (*d*) Injected sulphadiazine, which would have little action on the intestinal bacteria, was just as effective as that orally administered in raising the aneurin levels. This experiment also excluded the possibility that the effect of sulphadiazine was on the absorption of the orally administered aneurin.

(3) *Acetylation.* Sulphonamides are extensively acetylated *in vivo*, particularly in the liver. Klein & Harris (1938), James (1939, 1940), Martin & Rennebaum (1943) and Kinnunen (1946) have shown that this acetylation is dependent upon the supply of pyruvate or other intermediary metabolites, which, according to widely accepted views, are converted into a C_2 compound *in vivo*. According to Kinnunen (1946), it is this C_2 compound which is responsible for the acetylation, and sulphonamides compete with oxalacetate for the C_2 compound. Thus malate, which is converted to oxalacetate, reduces the

acetylation of sulphonamides, while the latter inhibit the formation of citrate from oxalacetate. Martin & Rennebaum (1943), Lipmann & Kaplan (1946) and Kinnunen (1946) have all implicated aneurin in the acetylation, since aneurin-deficient animals acetylate sulphonamides poorly, and this is increased both *in vivo* and *in vitro* by the injection of aneurin or by the addition of cocarboxylase to liver preparations (cf. also the effect of aneurin on acetylcholine synthesis; Mann & Quastel, 1939). Kinnunen (1946) considers that the effect of aneurin deficiency is due to a reduction in the amount of C_2 compound formed.

It is necessary, then, to consider the possibility that the effect of sulphonamides on the aneurin economy described in the present paper is a consequence of their acetylation. According to Kinnunen (1946), the reaction limiting both the acetylation of sulphonamides and the condensation of the C_2 compound with oxalacetate would be, in the aneurin-deficient animal, the conversion of pyruvate into the C_2 compound. It is difficult to see why the introduction of the sulphonamide, which competes with the oxalacetate for the C_2 compound, should affect the conversion of pyruvate into the C_2 compound. It would only reduce the rate of the Krebs cycle and this to only a very slight extent, since 100 times as much carbohydrate as sulphonamide was fed to the animal. Thus the acetylation of sulphonamide provides no explanation for the lower blood-pyruvate and higher tissue-aneurin levels found in the present investigation. Moreover, PAB, which is acetylated in the same way as sulphonamides (Doisy & Westerfeld, 1943), had no effect whatever on the tissue-aneurin levels (Table 3). Table 7 shows also that the effect on aneurin economy is not common to all sulphonamides, although these are all acetylated in essentially the same manner (Kinnunen, 1946).

(4) *Inhibition of aneurin-containing enzymes.* Sulphadiazine and sulphamerazine, which were the only two sulphonamides affecting aneurin economy, are chemically related to aneurin. It seemed possible then that these drugs displaced aneurin from some enzyme containing aneurin or a derivative as prosthetic group. There is, in fact, some evidence that sulphonamides can act in this way. Sevag, Shelburne & Mudd (1942, 1945) have shown that certain sulphonamides inhibit yeast and bacterial carboxylases and that this inhibition is reversed by cocarboxylase. However, there are a number of reasons which indicate that such an inhibition of an aneurin-containing enzyme cannot be the explanation of the present results: (1) Sevag *et al.* (1942) found that sulphathiazole was much more inhibitory on carboxylase than other sulphonamides, whereas sulphathiazole had no effect in the present experiments; (2) Sevag *et al.* (1942) required very much higher concentrations of sulphonamide than would be possible in mammalian tissues; (3) although such a displacement of aneurin from an enzyme might lead to raised tissue levels of aneurin, it is difficult to see why probably the most important *in vivo* function of an aneurin-containing enzyme, viz. the metabolism of pyruvate, should apparently be enhanced. Frohman & Day (1949) have found that injection of hydroxyaneurin (oxythiamine), an aneurin antagonist, into rats caused an increase both of the urinary excretion of aneurin and of the blood pyruvate.

None of the above explanations satisfactorily dealt with the finding that the increased tissue levels of aneurin were accompanied by lower blood-pyruvate levels and in the human experiment by an increased urinary excretion. Sulphadiazine acts, in all these three respects, by nullifying the effects of the aneurin deficiency, i.e. sulphadiazine has a true aneurin-sparing action on the animal. In general, there are three ways by which the drug could have this effect: (1) by replacing aneurin in some enzyme system; (2) by preventing the destruction of aneurin; (3) by altering the metabolism in such a way as to decrease the animal's requirement of aneurin. The first explanation is difficult to reconcile with the known specificity of aneurin in biological systems. While it is not possible to exclude the second explanation, it is rather unlikely since it implies that at least half of the aneurin provided to animals not fed the drug is destroyed. The third explanation is believed to be the most likely and will be considered in greater detail. Since the drug is effective in relatively low concentrations, its effect on metabolism is most likely due to an interference with some catalytic mechanism, rather than to direct intervention as a metabolite in the metabolic processes, e.g. by competing with oxalacetate for the C_2 compound. The delay in reaching the maximum excretion of aneurin with the human subject given sulphadiazine (Slater, 1946) rather suggests that the action of the drug might be indirect. Although sulphanilamide has some depressing effect on the respiration of spermatozoa (Knodt & Salisbury, 1946), the sulphonamides which have been tried either *in vivo* or *in vitro* do not appear to have any marked effect on the respiratory enzymes in tissue preparations (Rerabek, 1943; Anderson, Pilgrim & Elvehjem, 1944), except glucose-6-phosphate dehydrogenase, which is inhibited by a number of sulphonamides (Altman, 1946). However, sulphadiazine or sulphamerazine were not used in the studies of Rerabek or of Anderson *et al.* (1944). Altman (1946) found that sulphadiazine was considerably less inhibitory than sulphanilamide, sulphapyridine or sulphathiazole. Thus, none of these studies provides any clue as to the mechanism of the effects described in the present paper. Similarly, these cannot be connected with the inhibition of carbonic anhydrase, since sulphonamides substi-

tuted in the sulphonamide group do not inhibit this enzyme (Mann & Keilin, 1940).

Mackenzie & Mackenzie (1943) and Astwood, Sullivan, Bissell & Tyslowitz (1943) have shown that certain sulphonamides, when fed to rats, produced a remarkable enlargement of the thyroid gland, which was prevented by thyroxine. The thyroid hypertrophy and hyperplasia were accompanied by a marked drop in the basal metabolic rate (20 % after 14 days). Sulphadiazine was by far the most effective sulphonamide. Franklin & Chaikoff (1944) have found that sulphonamides interfere directly with the conversion of inorganic iodide to diiodotyrosine and thyroxine by surviving slices of thyroid tissue.

This effect of sulphadiazine on the weight of the thyroid was confirmed by the experiment described in Table 7. Sulphamerazine had the same effect. In the doses employed, sulphapyridine had a suggestive but statistically non-significant effect while sulphanilamide and sulphathiazole were ineffective. It was striking that the only two drugs which had this marked effect on the thyroid were also the only two which caused raised tissue-aneurin and lowered blood-pyruvate levels. This suggests the possibility that the effect of sulphadiazine and sulphamerazine on the aneurin economy is a consequence of the lowered basal metabolic rate caused by the effect on the thyroid. This hypothesis should be tested by trying the effect on the aneurin and pyruvate levels of other substances, such as thiourea, which also decrease the synthesis of thyroxine.

The incorporation of both liver powder and PAB (0·01 %) into the diet did not prevent the thyroid enlargement (this was also found by Mackenzie & Mackenzie, 1943) or the increased tissue aneurin produced by sulphadiazine. A much larger percentage of PAB (0·3 %, equimolar with the sulphadiazine) was also found to have no effect on the amount of aneurin in the tissues. This lack of effect of such a large dose of PAB as well as the fact that not all sulphonamides have the two effects is a clear indication that the reaction with which sulphadiazine is concerned in animal tissues is very different from that responsible for the bacteriostatic action of sulphonamides. In fact, none of the effects of sulphonamides on animals appear to be affected by PAB, with the exception of those toxic symptoms which are caused by the suppression by the sulphonamide of the bacterial synthesis of a vitamin in the intestine (Black, Overman, Elvehjem & Link, 1942; Kornberg, Daft & Sebrell, 1944; Petering, Delor & Murray, 1947).

It is interesting that sulphadiazine did not have any effect on the riboflavin economy of the rat. This suggests that the supply of riboflavin is much less critical than that of aneurin for the metabolism of pyruvic acid.

It is noteworthy that those drugs which had the effect on the thyroid and on the aneurin economy also caused a small but significantly lower kidney weight (Table 7). This may be a reflection of the improved aneurin nutrition of these animals, since Zucker & Zucker (1946) found that the kidneys of vitamin B-complex deficient rats were 50 % overweight. Usually, the liver of rats fed sulphadiazine was about 10 % heavier than the control rats. The reason for this is not known, but it may be connected with the acetylation which occurs in the liver (cf. Kinnunen, 1946).

The present experiments give no support to the suggestions of several authors (reviewed by Kinnunen, 1946) that the administration of sulphonamides increases the aneurin requirement (except when intestinal synthesis of aneurin provides a significant proportion of the total intake; Najjar & Holt, 1943). In fact, the results show that most sulphonamides have no effect on the aneurin economy, whilst those which do have an effect, viz. sulphadiazine and sulphamerazine, decrease rather than increase the requirement.

SUMMARY

1. A method of determining aneurin in rat liver and rat carcasses is described.

2. An approximately linear relationship was found between the aneurin content of the liver and the dietary intake of aneurin over the range 7·6–20·7 μg./day.

3. The inclusion of 0·55 % sulphadiazine in a diet moderately low in aneurin brought about the following changes in young rats compared with controls fed the same diet without the drug: (1) the aneurin content of the liver was about 60 % greater; (2) the aneurin content of the whole rat less liver was also greater; (3) the blood pyruvate was lower by about 30 %; (4) the kidney weight was 10 % less; (5) the liver weight was 10 % greater; (6) the thyroid was three times as heavy.

4. Injections of the drug had the same effect as oral administration.

5. Sulphamerazine behaved in the same way as sulphadiazine, but sulphanilamide, sulphapyridine, sulphathiazole, succinylsulphathiazole and 2-amino-4-methylpyrimidine, in equimolar amounts, did not show this effect.

6. *p*-Aminobenzoic acid (in an equimolar amount) did not behave like sulphadiazine, nor did it prevent the effects of the sulphonamide when the two were fed together. The effect of sulphadiazine was also not influenced by the inclusion or absence of folic acid and biotin in the diet.

7. Sulphadiazine had no appreciable effect on the riboflavin content of the tissues.

8. Inclusion of sulphadiazine in a diet low in aneurin prevented the development of typical

aneurin-deficiency symptoms, although it did not prolong the life of the rats.

9. It is concluded that sulphadiazine has a true aneurin-sparing action on the animal. This cannot be explained on the basis of (1) an effect on the renal threshold of aneurin; (2) change of bacterial flora in the intestine with altered vitamin synthesis; (3) acetylation of sulphonamide; (4) inhibition of intracellular enzymes containing aneurin.

10. It is thought probable that the sulphadiazine, by interfering with some catalytic mechanism, alters the metabolism in such a way as to decrease the animal's requirement of aneurin.

11. It is suggested that this alteration results from the inhibition of the synthesis of thyroxin caused by certain sulphonamides.

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The Nature of Pregnanediol-like Glucuronide

By B. W. L. BROOKSBANK* and G. A. D. HASLEWOOD
Guy's Hospital Medical School, London, S.E. 1

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A method was recently described (Bisset, Brooksbank & Haslewood, 1948) for the estimation of a fraction of urinary glucuronide with solubilities resembling those of pregnanediolglucuronidic acid and its salts. Analyses of normal men's urine and urine of women in the 'follicular' phase of the menstrual cycle gave values (expressed as pure sodium pregnanediolglucuronidate, Na*PG*) of glucuronide output higher than those obtained for Na*PG* by other workers. The total glucuronide fraction (entrained on barium phosphate) was designated 'pregnanediol-like glucuronide (*PLG*)', and we decided to extract the material in larger quantities and to attempt to determine its precise chemical nature.

Table 1 lists steroids which have already been obtained from glucuronide mixtures or from single glucuronides extracted from the urines named. The presence of conjugated 17-ketosteroids in urinary glucuronide fractions was suggested by the results of Allen & Viergiver (1941). In addition, Mason (1948) has described experiments which indicate the existence in the urine examined of several more steroids as glucuronide conjugates. He subjected *n*-butanol extracts of urine (that of a woman with an adrenal cortical tumour), freed of free steroids, to enzymic hydrolysis, using rat-liver powder known to contain β-glucuronidase and no sulphatase. The following free steroids were liberated partially or completely in this way: androsterone, aetiocholane-3α-ol-17-one, aetiocholane-3α:17β-diol (referred to by Mason (1948), as a 3:17α-diol), pregnane-3α:20α-diol, a compound T, $C_{19}H_{32}O_2$, m.p. 207–209°, and a compound $C_{21}H_{34}O$, m.p. 145°. These experiments do not, of course, exclude the possibility that the compounds mentioned were liberated from some unknown type of conjugate.

Table 1. *Steroids isolated from urinary glucuronides*

Steroid	Urine	Authors
Oestriol	Human pregnancy	Cohen & Marrian, 1936
Pregnane-3α:20α-diol	Human pregnancy	Venning & Browne, 1936
Pregnane-3α:20α-diol	Human pregnancy	Sutherland & Marrian, 1947
Pregnane-3α:20α-diol	Human female non-pregnancy	Venning, Henry & Browne, 1937
Pregnane-3α:20α-diol	Human male	Westphal, 1944
Pregnane-3α-ol-20-one	Human pregnancy	Marrian & Gough, 1946
Pregnane-3α:17α-diol-20-one	Human female pseudo-hermaphrodite	Mason & Strickler, 1947
Pregnane-3α:17α:20α-triol	Human female (adrenal cortical hyperplasia)	Mason & Kepler, 1945

We now describe the isolation from urine of a glucuronide fraction resembling as closely as possible the *PLG* estimated by the procedure of Bisset *et al.* (1948), and the investigation of material resulting from its hydrolysis. Part of the work has already been reported in a preliminary communication (Brooksbank & Haslewood, 1949).

We decided to use bacteria for the hydrolysis of the glucuronide mixture after preliminary experiments had suggested that acid hydrolysis led to extensive destruction. Concurrent research on the occurrence of glucuronide-splitting ability in micro-organisms had demonstrated that certain strains of *Staphylococcus albus* are particularly active in this respect (Barber, Brooksbank & Haslewood, 1948), and one of these active strains was employed for the large-scale hydrolysis of *PLG*.

METHODS

Melting points are uncorrected. The micro-analysis was done by Drs Weiler and Strauss, Oxford.

Abbreviations. Na*PG*(V) = a crystalline specimen of glucuronidate, prepared by the method of Venning (1937, 1938); Na*PG*(M) = purified glucuronidate, freed from ketonic material (Sutherland & Marrian, 1947); Na*PG* = anhydrous Na pregnanediolglucuronidate; *PLG* = pregnanediol-like glucuronide, i.e. strictly, material estimated by the method of Bisset *et al.* 1948; the term is, however, used to denote the material extracted and investigated on the large scale.

Light petroleum used was of b.p. 40–50°. Al_2O_3 for adsorption was obtained from Hopkin and Williams Ltd.

Estimation of glucuronide in crude PLG, etc.

The estimation of glucuronide entrained on Ba phosphate precipitates, of glucuronide in crude batches of *PLG* recovered from these precipitates and from chromatographic columns was performed by heating directly with naphthoresorcinol and HCl as described by Bisset *et al.* (1948). Thus, for the estimation of glucuronide entrained on Ba phosphate, samples (in duplicate) expected to contain not more than 0·2 mg. of glucuronide (as Na*PG*) were weighed

* Now at St Thomas's Hospital Medical School, London, S.E. 1.

out in test tubes (15 × 2 cm.) and dissolved in 2 ml. of 2N-HCl. To each tube were then added water (1·4 ml.), 0·05M-$K_3Fe(CN)_6$ (0·1 ml.), 9N-HCl (1·5 ml.) and 2 ml. of aqueous 0·025M-naphthoresorcinol solution (freshly made up and filtered). Colour development and measurement were then carried out as previously described. For the estimation of glucuronide in crude *PLG* samples, 2–4 mg. of the sample were weighed (in duplicate) and dissolved in water (20 ml.). This usually gave a solution of which 1·0–1·4 ml. contained the amount of glucuronide (0·05–0·2 mg.) necessary for accurate measurement by our modification of the naphthoresorcinol reaction, using the quantities of reagents given above.

Assay figures quoted throughout are in terms of pure anhydrous Na*PG*. However, *PLG* samples were always hygroscopic, apparently becoming more so as they were purified, and it was therefore not possible to weigh the samples accurately. Errors due to uptake of water were minimized by allowing the samples to stand 10–15 min. between removal from the vacuum desiccator in which they were kept and weighing. Duplicate assays then rarely differed by more than 10%. Nevertheless, the hygroscopic nature of *PLG* samples made it impossible to determine their true glucuronide content precisely. Even allowing for the fact that the molecular weights of a considerable proportion of the *PLG* constituents (e.g. androstenylglucuronide) may be lower than that of Na*PG*, the actual figures of percentage purity quoted probably err on the low side on account of the uptake of water.

In the colorimetric estimations, Na*PG*(V) solution was sometimes used as standard; this was in turn standardized against Na*PG*(M). More usually the grey solution described by Thomson (1946) was used as artificial standard, as it was more stable than Na*PG*(V) solutions.

Preparation of PLG

Collection and extraction of urine. Fresh unpreserved pooled men's urine was collected in lots of 4·8 l. Each lot was shaken with Zn acetate (96 g.), filtered, and 4 l. of the filtrate extracted with *n*-butanol (2 l., purified and tested for glucuronide-destroying impurities as previously described by Bisset *et al.* 1948). In order to save time and solvent, only one extraction with butanol was performed on each batch of urine, and the same butanol (with the volume adjusted with fresh butanol to 2 l. after each use) was employed for a total of five extractions. The butanol extract, which then represented 20 l. of urine, was washed with 0·2N-NaOH (900 ml.) and then with 2% (w/v) NaCl solution (2 × 180 ml.). The butanol was then evaporated to dryness *in vacuo*, ethanol being used to remove the last traces.

The residue contained usually about 100 mg. of *PLG* at this stage. This was determined on a number of batches by dissolving the residue in water to give a glucuronide concentration judged to be of the order of 0·1–0·2 mg./ml. and performing quantitative entrainment and glucuronide estimations on suitable samples (Bisset *et al.* 1948).

Entrainment of PLG. The residue from butanol extraction of 20 l. of urine was dissolved in water to give a concentration of *PLG* which was near to that obtaining in the diluted filtrate (*F*) used for entrainment in the quantitative procedure (Bisset *et al.* 1948), but which would give a consistent and fair yield of *PLG* by entrainment. In preliminary work with residues from 20 l. of urine, 80 times the quantities of water and of precipitating reagents used in the small-scale quantitative procedure (the corresponding residue of which is equivalent to 250 ml. of urine) gave unsatisfactorily low and inconsistent yields of *PLG*. We therefore used volumes of water and of precipitating reagents 50 times those used on the small scale. The butanol residue was dissolved in 1 l. of water and treated with 250 ml. each of $BaCl_2$ (0·5M) and Na_2HPO_4 (0·2M, containing 50 ml. N-NaOH/l.).

To ensure complete solution of the *PLG* the extract was warmed to 95° and cooled to room temperature before entrainment. If the thymol-salicylic acid mixture had been used as preservative, as was done with women's urine, much of it came through at this stage. The aqueous extract was in these cases washed with ethyl ether (separated by centrifugation) before entrainment of *PLG* was carried out (*PLG* was insoluble in ethyl ether).

After entrainment, the Ba phosphate precipitate was filtered off under suction and washed by stirring with acetone (300 ml., redistilled over CaO). The precipitate was then sucked dry and kept *in vacuo* over H_2SO_4.

Recovery of PLG from barium phosphate precipitates. Before the *PLG* was removed from the Ba phosphate it was further purified by extracting batches of 20–23 g. of Ba phosphate precipitate, each carrying about 200 mg. of *PLG*, with absolute ethanol for 2 days in a Soxhlet apparatus. Estimations on the residue from the ethanol used in these extractions never showed the presence of more than traces of glucuronide.

In the initial work the *PLG* was recovered from the Ba phosphate by exhaustive Soxhlet extraction with water, followed, after the addition of Na_2SO_4 solution to precipitate Ba^{++} ions, by extraction of the water with butanol. However, at least 4 days' extraction of the precipitate was necessary, and losses of up to 20% of the *PLG* were experienced. For this reason, the much quicker procedure was applied of dissolving the Ba phosphate in acid (for 20–23 g. batches, 150 ml. of N-HCl, quickly diluted to 0·5N) and extracting the acid solution without delay with purified *n*-butanol (120, 60 and 60 ml.). The butanol was washed once with 25 ml. of 2% (w/v) NaCl solution and shaken vigorously with excess solid $NaHCO_3$. The mixture was left overnight, washed twice with 25 ml. of water, filtered and evaporated to dryness at 50–55°/20 mm. Hg. The dry amorphous residue was dark brown and contained a small amount of inorganic material. It was extracted with warm methanol (30–40 ml., redistilled over KOH) and the methanol solution transferred to a weighed test tube and evaporated at 50–55° under a stream of N_2. The residue was scratched with 1–2 ml. of ice-cold dry acetone, which was filtered off and discarded. The acetone removed a small quantity of impurities and, if absolutely dry, negligible amounts of glucuronide. The acetone-insoluble material was collected and kept *in vacuo* over H_2SO_4; it was a concentrated preparation of crude *PLG*. From men's urine, about 8 mg. of this produce per litre were obtained. It assayed at 45–55% of glucuronide (as Na*PG*). Thus about 4 mg. of pure *PLG* (as Na*PG*) were obtained per litre of urine.

PLG from women's urine. Three 20 l. batches of women's urine were collected, in Winchester bottles each containing a mixture of powdered thymol (4 g.) and salicylic acid (2 g.) as preservative, from the 4th to 14th day of the menstrual cycle and pooled. The urine was extracted in the same way as described for men's urine. The amount of *PLG* was only about half that extracted from men's urine under the same conditions, and entrainment on Ba phosphate was carried

out on the 2nd and 3rd batches at double the concentration of residue in order to obtain a high yield of *PLG*; the butanol residue was dissolved in 500 ml. of water in the 2nd batch, and in 375 ml. of water and 125 ml. of 2% (w/v) NaCl solution in the 3rd batch, with the subsequent addition of 125 ml. each of the $BaCl_2$ and Na_2HPO_4 solutions in each case. The *PLG* derived in this way from women's urine therefore probably differed from that furnished by men's urine in containing extra, possibly non-steroidal, glucuronide such as is estimated in the quantitative procedure by the use of 4 ml. of extract at entrainment (Bisset *et al.* 1948). The final crude *PLG* obtained from 61 l. of urine weighed 311 mg. and assayed at the high figure of 69% of glucuronide. It did not differ in obvious properties from *PLG* obtained from men's urine.

Attempts at further purification of crude PLG. No very thorough study of the possibilities of purifying *PLG* by solvent partition or precipitation methods was made, but in a few experiments it was found that the following manoeuvres gave no appreciable purification and at the same time resulted in some loss of the material: extraction with hot ethanol or methanol (this was partially effective in purifying samples containing much inorganic material), precipitation from water with acetone, precipitation from methanol with ether (cf. Jayle & Libert, 1946), precipitation from methanol with acetone and precipitation from water with ethanol. In one experiment, extraction with $CHCl_3$ did seem to bring about considerable purification, although the glucuronide was appreciably soluble in $CHCl_3$.

We therefore attempted the purification of *PLG* by adsorption chromatography, hoping that as well as the removal of glucuronides from non-glucuronide material a partial separation of the glucuronide components of *PLG* might be effected. However, the glucuronide tended to be destroyed on the column (i.e. material giving a blue colour in the naphthoresorcinol reaction was 'lost' on the column). This destruction occurred more particularly when a high ratio of adsorbent to material adsorbed was used.

The progress of the column (no. 2, Table 5, see p. 41) which gave the purest *PLG* obtained is given in Table 2. In later columns more methanol and less 1:1 (v/v) butanol/methanol were used for elution, as the aim was to obtain large yields of reasonably pure glucuronide for hydrolysis experiments. Ethanol was relatively ineffective as an eluting solvent.

Hydrolysis of PLG

Acid hydrolysis. This was attempted with dilute HCl at room temperature and also with heating. No crystalline substances could be easily isolated, and the ether-soluble products appeared to have been partially decomposed.

Bacterial hydrolysis. A strain of *Staph. albus* known to have glucuronide-destroying activity was selected for large-scale work. A preliminary experiment was performed on Na*PG*. Na*PG*(V) (100 mg., equivalent to 97·1 mg. of Na*PG*) was dissolved in water (40 ml.) and the solution added to Lablemco peptone broth (200 ml.). The mixture was sterilized by autoclaving, seeded with the organism, and incubated for 1 week at 37°. After autoclaving, the mixture was filtered, and the filter paper carrying the precipitated material was extracted for 2 days with ethanol in a Soxhlet apparatus. The total ethanol-soluble material weighed 50·9 mg. From this material crystals were obtained (from the ethanol) which melted at 225–229°; mixed melting point with authentic pregnane-3α:20α-diol, 226–229°. No attempt was made to isolate all the pregnanediol (nor was any pregnane-3α-ol-20-one isolated), but if all the ethanol-soluble material was pregnanediol, this would represent 85% of the steroid moiety of the added conjugate. The absence of any conjugated glucuronide after incubation was confirmed by carrying out the glucuronide entrainment and estimation procedures on the residue from extraction of the filtered incubation mixture with butanol.

We therefore employed the same strain of *Staph. albus* to hydrolyse two batches of *PLG*. In the first experiment 151 mg. of the best chromatographed *PLG*, assaying at 76% and therefore containing 115 mg. of glucuronide (calculated as Na*PG*), were dissolved in water (40 ml.) and Lablemco peptone broth (200 ml.). This solution was sterilized by heating, seeded with an active strain of *Staph. albus* and incubated at 37°. At the end of incubation for 1 week it was again sterilized by autoclaving. (All the above bacteriological work was kindly executed by Dr Mary Barber at St Thomas's Hospital Medical School, London.)

Table 2. *Chromatogram of PLG from male urine*

(103·3 mg. of solid, containing 52·6 mg. of *PLG* (as Na*PG*) were put on to a column of Al_2O_3 (1 g.; approx. 5 × 50 mm.) with 1:1 (v/v) butanol/methanol.)

	Eluted with		Eluate		
Fraction	Vol. (ml.)	Solvent	Wt. (mg.)	Appearance	*PLG* content (as Na*PG*) (%)
1	25	1:1 (v/v) butanol/methanol	35·8	Yellow, glassy	7·9
2	20	1:1 (v/v) butanol/methanol	6·7	Nearly white, solid	28·9
3	20	1:1 (v/v) butanol/methanol	6·6	Nearly white, solid	60·1
4	30	1:1 (v/v) butanol methanol	4·2	Nearly white, solid	77·5
5	25	1:1 (v/v) butanol/methanol	5·0	Nearly white, solid	62·2
6	20	Methanol	13·3	Yellow, glassy (very hygroscopic)	92·7
7	100	Methanol	15·7	Brownish, amorphous	90·5
8	100	Methanol	3·1	Brownish, amorphous	92·2
9	100	Methanol	4·0	Brownish, slightly gummy	35·9
10	50	1% (v/v) acetic acid/methanol	7·6	Brown, glassy, gummy	30·2
		Total solid recovered	102·0 mg. = 98·7%		
		Total *PLG* recovered	48·3 mg. = 91·8%		

The autoclaved mixture, which contained a precipitate, was extracted with ether for 8 hr. in a continuous extractor. The ether extract gave a residue weighing approx. 30 mg., which appeared to contain some crystals. The aqueous layer was exhaustively extracted in a separating funnel with ethyl acetate. The ethyl acetate residue (approx. 40 mg.) was a gum, which showed a tendency to crystallize when treated with light petroleum. Both residues were combined (with ethanol) and together weighed 72·7 mg. All but an insoluble trace of this was dissolved in benzene and run on to Al_2O_3 (0·5 g.) in a column. Fraction 1 (eluted with 50 ml. benzene) weighed 37·4 mg., possessed a strong odour of musk and crystallized when treated with light petroleum. The second fraction (eluted with 50 ml. of 1:1 (v/v) ether/benzene) weighed 7 mg. and was crystalline and slightly odorous. Thereafter 1:1 (v/v) ether/benzene (50 ml.), ether (50 ml.) and ethanol (50 ml.) failed to elute any more material, so that about 28 mg. remained unaccounted for in this experiment. Fraction 1 was put on to Al_2O_3 (0·5 g.) in a column with, at first, approx. 50 ml. of light petroleum, in which it did not entirely dissolve. The bulk came through in the first fraction and was combined with a very small additional amount eluted with a further 50 ml. of light petroleum to give 22·7 mg. of very easily crystallizable material. This appeared to contain hydrocarbon-like substances, which were partly removed by the addition of a few drops of methanol to a solution in 1:1 (v/v) methanol/ethanol. The wax-like material separated out and was filtered off. The substance obtained by evaporation of mother liquor, which was the odorous, easily crystallizable material, was soluble in all the common organic solvents. It was finally crystallized from dilute acetone, and then from a very small quantity of light petroleum. Sublimation at 125°/0·3 mm. Hg gave material again recrystallized from light petroleum to yield long white needles, m.p. 143–143·5°, with a strong odour. (Found: C, 83·0; H, 11·3. Calc. for $C_{19}H_{30}O$: C, 83·2; H, 11·0%.) A sample of authentic androst-16-en-3α-ol (kindly sent to us by Prof. V. Prelog of Zürich; cf. Prelog & Ruzicka, 1944) resembled our substance closely and did not depress its melting point.

For the second bacterial hydrolysis 335 mg. of *PLG* assaying at 70% (235 mg. of glucuronide, calculated as Na*PG*) was dissolved in water (40 ml.), made up to 400 ml. with Lablemco peptone broth, and the sterilized mixture incubated for 6 days with the same strain of *Staph. albus* as in the previous experiment and finally autoclaved. A strong smell of musk was noted in the crude mixture even at this stage. The mixture was filtered, and the filter paper was extracted in a Soxhlet apparatus for 2 days with ether. The filtrate was shaken with ether in a separating funnel. The residue from the combined ether extracts weighed 114·2 mg.

To test for unchanged glucuronide conjugate, 10 ml. of the final aqueous filtrate were made up to 25 ml. with water, and the solution extracted with 10, 5 and 5 ml. of butanol. The butanol was washed with 0·2 N-NaOH (10 ml.) and 2% (w/v) aqueous NaCl (2+2 ml.) evaporated to dryness and the residue dissolved in water (10 ml.). Determination of glucuronide was performed on this aqueous extract both with and without entrainment. No glucuronide was entrained, showing that no unhydrolysed *PLG* remained in the incubated mixture. Estimation without entrainment showed an amount of 40 mg. of butanol-soluble glucuronide (as Na*PG*) in the total filtrate from the incubation mixture. Direct assay on the filtrate itself gave a figure of 122 mg., or 51·9% of the original amount of glucuronide (as Na*PG*); therefore, 32·8% of the total glucuronide present after bacterial hydrolysis was extractable with butanol.

The total ether-soluble material (114·2 mg.) extracted from the incubation mixture was dissolved in ethanol (2 ml., distilled over Na) and the solution refluxed for 1·5 hr. after the addition of A.R. acetic acid (0·2 ml.) and Girard's reagent T (0·1 g.). Some ethanol evaporated through a leak; further amounts of ethanol (2–3 ml.) and Girard's reagent T (0·1 g.) were added. The mixture was refluxed for a further 20 min., stoppered and left overnight. The non-ketonic fraction was extracted with ether after the addition of water to the mixture and neutralization to pH 7·6 (bromthymol blue); it weighed 76·0 mg. The ketonic fraction, extracted with ether from the aqueous solution after removal of non-ketonic material and acidification, weighed 11·0 mg. Therefore 27·2 mg. of the starting material were unaccounted for. The ketonic fraction was refluxed in ethanol (1 ml.) for 1 hr. with semicarbazide hydrochloride (20 mg.) and Na acetate ($CH_3COONa.3H_2O$; 30 mg.); no semicarbazone was precipitated.

The non-ketonic fraction (76 mg.) was warmed with 10 ml. of a solution of digitonin (1 g. in 90 ml. ethanol + 10 ml. water). The dried digitonide weighed 12·2 mg. It was decomposed with pyridine (1 ml.) and ether, and the ether-soluble material weighed 4·8 mg. This '3β-fraction' was semi-crystalline, but gave only a gum when sublimed in high vacuum. The digitonin non-precipitable fraction weighed 71·2 mg. Of this, 60 mg. were subjected to exhaustive fractionation on alumina. It was dissolved in benzene (5 ml.) and light petroleum was added until cloudiness just appeared; the solution was then poured on to a column of Al_2O_3 (3 g.). The solvent was then approx. 70% (v/v) benzene/light petroleum.

The progress of the column is shown in Table 3.

Fractions 1 and 2 were combined and separated again on a column, with 90% (v/v) light petroleum/benzene. The bulk came through in the first fraction as a gum, whilst 2·6 mg. of crystalline, musk-smelling material were eluted with 10 ml. 70% light petroleum/benzene.

Therefore, including fractions 3, 4 and 5, about 14 mg. of musk-smelling crystals were obtained from the 60 mg. of material. This sample (14 mg.) was recrystallized from 1:1 (v/v) acetone-water and then had m.p. 144–146°. It was shown by the following evidence to be androst-16-en-3α-ol as isolated in the previous experiment. Some of this material was sent, at his request, to Dr K. Dobriner of the Sloan-Kettering Institute for Cancer Research, New York, for determination of its infrared absorption spectrum. The curves (sent to us by Dr Dobriner) obtained with our material and that isolated from swine testes by Prelog & Ruzicka (1944) were identical.

The crystalline fractions 10, 11 and 12 (6·7 mg.) were combined and recrystallized from methanol containing a little water. The crystals melted at 224–226°; mixed melting point with pregnane-3α:20α-diol, 225–229°.

Enzymic hydrolysis experiments. These were carried out with the rat-liver preparation of Talbot, Ryan & Wolfe (1943). With this preparation (4 hr. at pH 5 in acetate buffer), Na*PG*(V) (50 mg.) gave pregnane-3α:20α-diol (17 mg.). Crude *PLG* (221 mg.) containing 115 mg. (as Na*PG*) of glucuronide gave about 10 mg. of crude androst-16-en-3α-ol, m.p. approx. 130°, with the characteristic smell. Further purification was made difficult by fatty material derived from the glucuronidase preparation.

Table 3. *Chromatogram of hydrolysed PLG from male urine*

(60 mg. digitonin non-precipitable material on 3 g. Al_2O_3: see text.)

	Eluted with		Eluate	
Fraction	Vol. (ml.)	Solvent	Wt. (mg.)	Appearance
1	10	70% (v/v) benzene/light petroleum	11·1	Gummy
2	10	70% (v/v) benzene/light petroleum	7·1	Crystalline, musk smell
3	10	70% (v/v) benzene/light petroleum	7·1	Crystalline, musk smell
4	10	70% (v/v) benzene/light petroleum	3·2	Crystalline, musk smell
5	10	70% (v/v) benzene/light petroleum	1·0	Crystalline, musk smell
6	10	80% (v/v) benzene/light petroleum	Nil	—
	10	90% (v/v) benzene/light petroleum	0·9	Gum
7	10	Benzene	Nil	—
	10	10% (v/v) ether/benzene	1·1	Gum
8	10	30% (v/v) ether/benzene	Nil	—
	10	50% (v/v) ether/benzene	1·5	Gum
9	10	70% (v/v) ether/benzene	Trace	—
10	10	Ether	0·5	White, crystalline
11	30	Ether	4·2	White, crystalline
12	20	Ether	2·0	White, crystalline
13	40	Ether	Trace	—
14	20	10% (v/v) ethanol/ether	5·2	White, amorphous
	20	20% (v/v) ethanol/ether	—	—
	20	30% (v/v) ethanol/ether	Nil	—
15	20	50% (v/v) ethanol/ether	Nil	—
	20	70% (v/v) ethanol/ether	Nil	—
	20	Ethanol	11·8	White, amorphous
16	10	Ethanol	Nil	—
	20	Ethanol	Trace	—
	20	Ethanol	1·5	White, amorphous
	20	Ethanol	—	—
17	20	Ethanol	Trace	—
		Total	58·2 mg.	

Hydrolysis of PLG from women's urine. The *PLG* from 61 l. of the urine of women in the 'follicular' phase of the menstrual cycle amounted to 215 mg. of glucuronide contained in material of 69% purity (see p. 38). The whole (311 mg.) was dissolved in 40 ml. of water, made up to 400 ml. with Lablemco peptone broth, seeded with the same strain of *Staph. albus* as used in the experiments described above and incubated for 6 days at 37°. The mixture was sterilized by heating for 20 min. at 95°, and filtered. The filter paper and filtrate were extracted with ether, but the combined ether residue weighed only 78 mg.

The ether-soluble material, partially dissolved in benzene, was put directly on to a column of Al_2O_3 (0·5 g.). Only the first fraction, which weighed 14·0 mg. and was eluted with 15 ml. of benzene, was investigated. It was semi-crystalline and smelt strongly of musk. This material was twice crystallized from dilute acetone and then melted at 149–150°, with previous softening. Recrystallization from about 1 ml. of light petroleum gave small white crystals which melted sharply at 160–161° and still smelt strongly. Yield, <1 mg. Material of similar melting point and properties was obtained by purification (sublimation, crystallization, chromatography) of the residue left on evaporation of the dilute acetone mother liquors.

RESULTS

Preliminary purification of PLG. Table 4 gives the yield of *PLG* at various stages in the extraction procedure as finally adopted. The figures for ethanol-soluble and -insoluble material refer to the yields obtained when the final butanol residue (see p. 37), instead of being taken up in methanol, was extracted several times with ethanol and the residual material then taken up in water or methanol. Batches *L* and *M* (combined) and subsequent batches were not fractionated in this way. These figures show some variation, but the average yields were satisfactory. The overall recovery of 85·5% of the *PLG* from the barium phosphate precipitate may be noted. The loss of Na*PG* by butanol extraction of pure aqueous solutions was about 16% (Bisset *et al.* 1948), and it is likely that the loss of *PLG* at this stage was at least of the same order; therefore, losses due to hydrolysis or any other cause were probably negligible. The average yield, in fifteen experiments, of *PLG* (as obtained on barium phosphate under the earlier

Table 4. *Yields of PLG from men's urine at various stages in the extraction procedure*

(For details of procedure, see p. 37. All weights and percentages of glucuronide in *PLG* are expressed as Na*PG*.)

Urine (batch of 20 l.)	*PLG* in butanol residue (mg.)	Wt. of Ba phosphate ppt. (g.)	*PLG* entrained on Ba phosphate ppt. (mg.)	Fraction	Acetone-insol. *PLG* (final residue) from batches of Ba phosphate ppt.		Percentage of entrained *PLG* recovered as acetone-insol. final residue
					PLG assay (mg.)	Purity (%)	
A	76·8	10·91	63·2	—	No systematic figures available as these lots were pooled and used in exploratory extraction experiments		
B	82·4	11·34	72·7	—			
C	75·0	10·97	52·3	—			
D	82·9	11·24	69·8	—			
E	90·0	13·06	78·3	—			
F	80·1	11·45	70·1	Ethanol-sol.	88·7	50·2	79·2
G	81·8	10·90	71·7	Ethanol-insol.	23·6	18·9	
H	—	11·14	51·3	Ethanol-sol.	53·7	48·6	70·8
I	—	11·54	66·5	Ethanol-insol.	29·7	21·6	
J	—	11·49	98·9	Ethanol-sol.	112·3	52·7	78·1
K	—	11·73	76·1	Ethanol-insol.	24·3	19·0	
L	—	11·35	89·4	Ethanol + water sol.	139·2	35·0	91·3
M	—	11·46	63·1				
N	—	11·83	93·4	Methanol-sol.	195·4	51·2	99·8
O	—	11·58	102·5				
P	—	11·43	122·9	Methanol-sol.	180·5	53·7	83·7
Q	—	11·78	92·7				
R	—	11·64	95·3	Methanol-sol.	157·3	50·7	88·4
S	—	10·06	82·5				
Mean yields/l. of urine	4·1	0·57	4·0		3·6		

Table 5. *Recovery of PLG from men's urine on alumina columns*

(1 g. Al_2O_3 used except where stated otherwise.)

Column	Material put on		Material recovered		*PLG* assaying above 69% (as Na*PG*)	
	Total solid (mg.)	Total *PLG* (as mg. Na*PG*)	Wt. *PLG* recovered (as mg. Na*PG*)	Percentage *PLG* recovered	Total wt. (mg.)	Average assay of samples (%)
1	101·6	54	26	48	17·5	72
2	103·3	53	48	91	36·3	90
3	99·8	51	43	84	36·6	73
*4	300·7	153	97	63	—	—
5	100·0	51	40	78	133·2	69
6	99·2	51	40	78		
7	99·1	51	41	80		
8, 9, 10	300·8	119	107	90	—	—
11, 12, 13	98·4	55	56	102	62·3	69
	199·8	111	86	77	115·1	75
14, 15, 16	309·9	163	133	82	151·2	71
*Na*PG*(V)	100·5	98	Approx. 22	Approx. 22	—	—

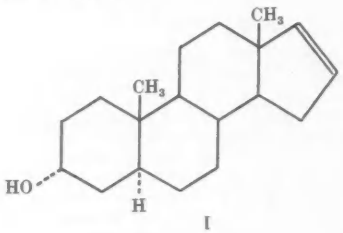
* 10 g. Al_2O_3.

'dilute' entrainment conditions) from 1 l. of men's urine extracted thoroughly with full quantities of fresh butanol, was 6·4 mg. Our time-saving procedure of extracting five 4 l. lots of urine with the same single lot of butanol (2 l.) gave an average yield of 4·0 mg./l. at the same stage (Table 4), and therefore entailed a loss of about 38 % of the total extractable *PLG*.

Purification of PLG on alumina columns. The results obtained on all but the first, exploratory, column are summarized in Table 5. Since the loss of *PLG* on column 4 was large, and no high-assay glucuronide was obtained from it, the use of only 1 g. of Al_2O_3 for approx. 100 mg. of *PLG* was continued in later experiments; the last three groups of columns are bracketed together as they were treated simultaneously in parallel. Column 11 was a separation of the main middle fraction (assaying at approx. 60 % as Na*PG*) from columns 8–10. The Na*PG*(V) column was a failure for no reason that was ascertained.

These results indicate that an average of 79 % of *PLG* put on the columns was recovered. It was apparent that no clear-cut separation of the components of *PLG* could be attained, particularly as a second attempted separation failed to effect more than a slight further purification of the glucuronide material.

Nature of PLG from men's urine. This clearly contained androst-16-en-3α-ol (I) or some substance which was converted into it under very mild conditions, and also pregnane-3α:20α-diol. Small amounts of ketonic material and of digitonin-precipitable substances were present.



I

DISCUSSION

Men's urine

Since the androstenol was isolated without the use of bacteria for hydrolysis, it was presumably not a bacterial artefact. The high concentration of glucuronide in the purer specimens of *PLG*, together with the absence of smell in these specimens, made it appear very probable that the androstenol was, in fact, present as a glucuronide in the original urine, although *PLG* was a hygroscopic gum and no crystalline glucuronide could be isolated.

It is much more difficult to answer the questions: (i) what proportion of *PLG* is the glucuronide of androst-16-en-3α-ol? (ii) to what extent is the androstenol estimated in a quantitative *PLG* determination? There is no doubt in our minds that the chief constituent of the purified *PLG* from the men's urine which we have handled was in fact the androstenylglucuronide, and indeed the systematic examination of hydrolysed *PLG* resulted in the isolation of only one other crystalline substance (pregnanediol) in small amount. However, the hygroscopic nature of *PLG* and the difficulty of reproducing samples of defined purity (as measured by the glucuronide assays) make it impossible to say more than that it is our belief that perhaps 50 % or more of our purest *PLG* probably consisted of sodium androst-16-en-3α-ylglucuronidate. An answer to the second question involves a consideration of the degree to which the larger-scale preparation of *PLG* reproduces the conditions of the quantitative assay previously described, together with some assessment of the extent to which this assay estimated the androstenylglucuronide. A comparison (see p. 37) of the quantitative assay with the preparative process now described shows that in the latter large losses of glucuronide occurred on entrainment, etc., although entrainment was in fact carried out from more concentrated urine extracts than were used in the quantitative *PLG* estimation. Thus the preparative process was at best only very roughly a reproduction on a larger scale of the assay, and deductions from it apply only to a limited extent to *PLG* estimations. Furthermore, the losses incurred on entrainment of the androstenylglucuronide are quite unknown and could not be estimated until a pure sample of this substance is available.

It therefore seems impossible to say more than that, since androst-16-en-3α-ylglucuronide is a major constituent of *PLG* from men's urine, and since the solubilities of this latter mixture are undoubtedly very similar to those of pregnanediolglucuronide, a *PLG* estimation in male urine does include a substantial contribution from the androstenylglucuronide present.

Women's urine

It was not possible, on the basis of our results, to say that the female 'follicular' urine examined by us contained androst-16-en-3α-ol, although the strong musk-like smell of the appropriate fractions led us to suspect that this was the case. However, the crystalline material of m.p. 160–161° isolated still smelt strongly. Further purification of larger amounts of this material ought to enable one to decide whether it is a new musk-smelling substance or whether (as seems more probable) the smell was associated with traces of the androstenol present as an impurity.

In view of the previous isolation (Prelog & Ruzicka, 1944) of androst-16-en-3α-ol from swine testes, it may perhaps be tentatively assumed that this compound is also of testicular origin in the human male. If this is the case, further consideration will be needed concerning the origin of the musk-smelling material undoubtedly present in a conjugated form in women's urine.

SUMMARY

1. 'Pregnanediol-like-glucuronide' (*PLG*), isolated by a larger-scale modification of a previously described analytical procedure, has been purified and hydrolysed by use of (*a*) a strain of *Staphylococcus albus*, and (*b*) a crude glucuronidase preparation.

2. *PLG* from men's urine yielded, after bacterial hydrolysis, a little ketonic material, a little digitonin-precipitable material, a little pregnane-3α:20α-diol and, as chief product, crystalline androst-16-en-3α-ol. The androstenol was also isolated after enzymic hydrolysis.

3. *PLG* from women's 'follicular' urine yielded, in a preliminary experiment, a musk-smelling fraction from which material of melting point about 160° was obtained. It was not possible to prove the presence of the above androstenol.

4. The significance of these results is discussed.

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Some Observations on the Kinetics of Haemoglobin in Solution and in the Red Blood Corpuscle

By J. W. Legge and F. J. W. Roughton
Department of Colloid Science, and Molteno Institute, University of Cambridge

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The kinetics of the rapid reactions of oxygen and carbon monoxide with the oxygen-carrying pigments have been studied by the rapid-flow methods of Hartridge & Roughton (1923*a–c*, 1925, 1927), Millikan (1932, 1933*a*, *b*, 1936) and Roughton (1934*a–d*). For myoglobin (Mgb), Millikan found that the rate of combination of oxygen could be expressed by the equation

$$\frac{d[O_2Mgb]}{dt} = k'[O_2]\,[Mgb] - k[O_2Mgb], \qquad (1)$$

and furthermore that $k/k' = K$, the equilibrium constant of the reaction ($K = [O_2]\,[Mgb]/[O_2Mgb]$) as determined from the dissociation curve, which is in this case a rectangular hyperbola. These simple relations are to be expected on the basis of the Law of Mass Action, since the myoglobin molecule only contains one iron atom and thus reacts reversibly with one molecule of oxygen according to the equation

$$O_2 + Mgb \rightleftharpoons O_2Mgb. \qquad (2)$$

In the case of mammalian haemoglobin, the situation is much more complicated, since the molecule contains four iron atoms and reacts reversibly with four molecules of O_2 or CO. It is true that Hartridge & Roughton found that their kinetic results could, within the limits of error and range of their measurements, be described by an equation similar to (1), namely

$$\frac{d[\mathrm{XHb}]}{dt} = k'[\mathrm{X}]\,[\mathrm{Hb}] - k[\mathrm{XHb}], \qquad (3)$$

where [X] = concentration of dissolved O_2 or CO; [XHb] = concentration of oxyhaemoglobin or carboxyhaemoglobin in g. mol. of combined O_2 or CO/l.; [Hb] = concentration of reduced haemoglobin expressed in the same units as XHb.

The equilibrium results are, on the other hand, generally interpreted by some form of Adair's (1925) intermediate compound hypothesis according to which

$$\frac{y}{100} = \frac{[\mathrm{XHb}]}{[\mathrm{XHb}]+[\mathrm{Hb}]} = \frac{K_1p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4)}, \qquad (4)$$

where y = percentage saturation; p = pressure of X; K_1, K_2, ..., are the respective equilibrium constants of the reactions $Hb_4 + O_2 \rightleftharpoons Hb_4O_2$, $Hb_4O_2 + O_2 \rightleftharpoons Hb_4O_4$, etc.

Some attempts have recently been made to reconcile equations (3) and (4) (Wyman, 1948; Roughton, 1949), but there is clearly need of much further work in this field, both theoretical and experimental. One great difficulty is that many different sets of values of K_1, K_2, K_3, K_4 give a good fit between equation (4) and the experimental results, though, even so, it always appears necessary to assign a much higher* value to K_4 than to any of the other constants. This means that the last O_2 (or CO) molecule combines with much greater affinity than the preceding ones. Pauling (1935) has suggested that a similar but smaller 'interaction' occurs in the case of the lower intermediates, but the equation derived on his detailed hypothesis has recently been shown not to be valid for the best available dissociation curves (Roughton, 1943, 1949; Wyman, 1948; Roughton, Paul & Longmuir, 1949).

Several kinetic points arise out of these and other considerations:

(*a*) Since $K_4 = k'_4/k_4$, where k'_4 and k_4 are the velocity constants of the reaction

$$Hb_4O_6 + O_2 \rightleftharpoons Hb_4O_8,$$

then if K_4 is relatively large, either k'_4 must also be relatively large or k_4 relatively small. Calculation of the empirical velocity constants, k' and k, of equation (3) should therefore, at high values of y, show a rising trend in k' or a falling trend in k, or perhaps both. No such tests have hitherto been made above $y = 75\,\%$: in this paper analysis of present and earlier data shows that both these effects do in fact occur.

(*b*) In all previous reaction-velocity work, the haemoglobin before mixture has been either fully reduced or fully saturated. Tests should be made as to whether the results obtained with partially saturated haemoglobin as the starting point are in agreement: a discrepancy would indicate that when haemoglobin is partially saturated, the concentrations of the various intermediates, or their energy levels, differ appreciably according to whether the haemoglobin is in equilibrium or is in a state of change when the particular partial saturation occurs. Such observations might also throw light upon a peculiar result of Roughton (1934*d*), who found that, at pH 10 and 15°, CO combines twice as rapidly with haemoglobin just after its formation from oxyhaemoglobin by admixture with sodium dithionite ($Na_2S_2O_4$) as it does if the reduced haemoglobin is more than 2 sec. old. It is possible that the 'freshly' reduced haemoglobin might not have been quite completely reduced and that small traces of Hb_4O_2, Hb_4O_4, etc., might still have been present. In such an event, CO would have reacted with iron atoms adjacent to some which were already combined with O_2, this leading perhaps to a faster rate of combination than in the case of 'old' reduced haemoglobin in which no Hb_4O_2, etc., could have been present.

In this paper, tests are given on the effect of initial partial saturation of the haemoglobin, both on the rate of combination of CO with haemoglobin and on the rate of dissociation of oxyhaemoglobin in presence of $Na_2S_2O_4$, these two reactions being the most satisfactory for studies of this kind. Adult sheep haemoglobin was used, since it was the main source of material used in previous work, and furthermore it is not believed to split into submolecules, at the concentrations suitable for our experiments. The results were mostly negative, except perhaps in the dissociation of oxyhaemoglobin at neutral pH. Here, however, an interesting new phenomenon was discovered, the oxyhaemoglobin in the presence of $Na_2S_2O_4$ often appearing to dissociate in two phases, one fast and the other slow. The explanation is probably complex, and though not fully worked out, it has been felt worth-while to discuss the data in some detail, in view of the widespread use of $Na_2S_2O_4$ for reduction of oxyhaemoglobin and other substances of physiological interest.

(*c*) Recently, Nicolson & Roughton (1949) have made a much fuller analysis than was possible in Roughton's earlier paper (1932), of the rates of reaction of O_2 and CO with haemoglobin in red-cell suspensions as compared with the rates with haemo-

* Work now in progress by one of us (F. J. W. R.) suggests that the ratio of K_3 to K_4 may be higher than previously supposed.

globin in homogeneous solution. They believe that it is possible, by their methods, to compute approximately for the first time, the permeability of the red-cell membrane to O_2 and CO. For this purpose, new and more accurate data were required on the rates of the reactions of haemoglobin in solution and in the red cell. Such data were secured as a by-product of the main experimental projects of this paper, and a brief description of the actual experiments is accordingly included.

EXPERIMENTAL METHODS

General principle

The reagents are prepared in separate bottles (2·5–3·0 l. capacity) and driven thence, by compressed N_2 or air, through separate leads into a mixing chamber. From there the mixed fluid emerges into a glass observation tube, and the concentration of the various haemoglobin compounds in the streaming fluid at various points along the observation tube are determined by Millikan's (1932) differential photocolorimetric method (slightly modified). Such determinations, together with a knowledge of the rate of fluid flow down the observation tube and of the total concentration of each reagent, give the data required for measuring the velocity of the reaction.

Experimental details

Mode of preparation of the reagents

(*a*) *Oxygen-free water or buffer solutions.* A bottle was usually half-filled with solution, evacuated and rolled vigorously for 4 min. Nitrogen was then admitted to atmospheric pressure, the gas phase re-evacuated and the rolling repeated. Alternatively, the bottle was warmed to about 30°, evacuated until the solution boiled, rolled, re-evacuated and rolled again without admission of N_2.

(*b*) *Solution of* CO. The solution was first freed of O_2 as in (*a*). CO was then admitted to a suitable pressure and the bottle rolled therewith for 4 min. (or longer if the bottle was more than half full of liquid). The CO was either prepared from Na formate and conc. H_2SO_4, or drawn from a cylinder of compressed gas: in either case it was stored under pressure over alkaline $Na_2S_2O_4$, so as to remove traces of CO_2 and of O_2.

(*c*) *Solution of* O_2. The bottle was partially filled with solution as in (*a*), the air evacuated off, replaced with a suitable pressure of O_2 and rolled for 4 min. or longer. The concentration of dissolved O_2, or of CO in (*b*), was calculated from the composition of the gas phase, its pressure, the relative volumes of gas and liquid phases and the appropriate solubility coefficients.

(*d*) $Na_2S_2O_4$ *solution.* The standard concentrations used were 0·1 and 0·2 %, and were prepared by dissolving weighed amounts of the solid in the buffer solutions with minimal contact with air. $Na_2S_2O_4$ in solution develops acidity, and appropriate amounts of NaOH (as determined by the glass electrode) were also added to the buffers, so as to restore the latter to the stated pH values.

(*e*) *Reduced haemoglobin solution.* Defibrinated sheep blood was reduced by repeated evacuation and shaking while warm, as described by Forbes & Roughton (1931), the whole process taking 30–60 min. The required volume of reduced blood was then transferred anaerobically, via a mercury reservoir and burette, to the bottle containing O_2-free water, the final haemoglobin concentration being usually about 0·4 %, at which strength adult sheep haemoglobin shows no tendency to dissociate into submolecules. It was not found satisfactory to prepare reduced haemoglobin merely by adding $Na_2S_2O_4$, since, as previous work has shown, there is danger, on prolonged standing, of formation of variable amounts of choleglobin (Legge & Lemberg, 1941) and possibly of other compounds.

(*f*) *Oxyhaemoglobin solution.* This was prepared in several ways: (i) by adding a suitable volume (usually 1 in 30) of defibrinated sheep blood to aerated water; (ii) the reduced blood, prepared as in (*e*), was re-aerated by 15 min. rotation in a tonometer with air and then added to the aerated water; (iii) the reduced blood was added to 29 parts of water, previously equilibrated with O_2 at a partial pressure of 300 mm. Hg, the dissolved O_2 content of the water being sufficient to re-oxygenate the haemoglobin almost completely. The purpose of methods (ii) and (iii) was to control for possible changes in the haemoglobin which might occur during the reduction process described under (*e*).

(*g*) *Carboxyhaemoglobin solution.* This was only required for calibration purposes and was prepared by adding 1 part of defibrinated sheep blood, which had been rotated for 15 min. with CO at 1 atmosphere pressure, to 29 parts of water.

The special reagents required for the experiments on the red-cell suspensions are described in the section dealing with the red-cell results.

The reaction-velocity apparatus

The reagent bottles were connected via a tube of 5 mm. bore, with a tube of capillary bore in parallel, to either side of a two-way Perspex mixing chamber. The rate of flow could thus be varied about threefold, according to whether the 5 mm. bore tube or the capillary tube was open. The observation tube was first of the form shown in Fig. 1 *A*, but later when more extended times of reaction were required, other forms (Fig. 1 *B*, *C*) were used. Type *B* proved unsatisfactory for red-cell suspensions, since a rotatory motion was imparted to the fluid when it entered the enlarged part of the tube, thus resulting in a partial sedimentation of the red cells, easily visible in optical irregularities as the fluid left the enlarged part of the tube. The form of fluid motion in type *C* did not cause such irregularity. The driving pressures ranged from 20 to 40 cm. Hg and the rate of flow down the observation tube from 30 to 150 cm./sec. The mixing chamber and observation tube were attached to a vertical adjustable stand, by means of which different parts of the observation tube could be brought under examination.

Differential photocolorimetry

Light from a 6 V. 12 W. projection lamp, fed by two 2 V. accumulators in series (for greater stability the lamp was thus run below its maximum brightness) was focused on to the observation tube. The beam then passed through a red-green filter, as described by Millikan (1932), and thence on to an EEL differential selenium cell (Evans Electroselenium Ltd, Harlow, Essex). The photocurrent generated by the latter was recorded by a Pye moving-coil galvanometer (deflexion 450 mm./μa. at 4 m. scale distance, time of

indication about 1·5 sec.). At each position of the observation tube, the light beam was so focused and the red-green filter so adjusted that (i) the galvanometer deflexion was as near zero as possible (small residual deflexions being balanced off by a compensating potentiometer), (ii) the change from oxyhaemoglobin (or carboxyhaemoglobin) to reduced haemoglobin caused a deflexion of 50–120 mm. A reading was then taken with reduced haemoglobin flowing through the tube, then with the reacting fluid, and finally with oxyhaemoglobin or carboxyhaemoglobin standard. Errors due to drifts in light intensity and/or photocell sensitivity are thus much reduced.

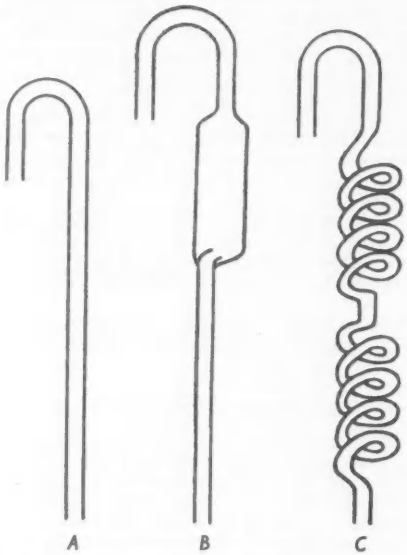

Fig. 1. Various forms of observation tube in this paper. *A*, general form; *B*, form for special study of processes with a rapid phase and slow phase; *C*, form for study of red-cell suspensions.

Under our conditions, as in Millikan's, the proportions of the various haemoglobin compounds present (i.e. the 'percentage saturation') were obtained by linear interpolation from the end-point readings. That this procedure was correct to within 1–2% saturation was checked, both by Millikan's theoretical method and also by means of haemoglobin solutions of known percentage saturation.

RESULTS AND DISCUSSION

1. *The rate of combination of dissolved carbon monoxide with reduced haemoglobin*

A. *Haemoglobin solutions.* In Fig. 2*A*, *B* is shown the rate of combination of a solution of CO in water (0·22 mM) with (*a*) fully reduced Hb (0·25 mM), and (*b*) the same haemoglobin containing initially 19% COHb. The haemoglobin solution was made up of 1 part blood + 29 parts of 0·1 M-ammonium chloride – 0·05 M-ammonia buffer mixture, pH approx. 9·5, temp. 17°. The rates of reaction do not differ materially provided allowance is made for the higher total concentration of CO, dissolved and combined in (*b*).

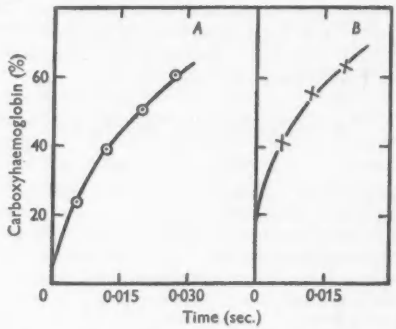

Fig. 2. *A*, rate of combination of CO with reduced Hb; *B*, rate of combination of CO with 19% COHb + 81% reduced Hb.

If α = total concentration of CO, dissolved and combined, β = total concentration of haemoglobin expressed in gas-combining capacity of O_2 or CO, z = concentration of COHb at time t, then equation (3) may be written

$$\frac{dz}{dt} = l'(\alpha - z)(\beta - z) - l, \qquad (4)$$

the constants k' and k being replaced by l' and l, so as to distinguish the process as a CO reaction. The

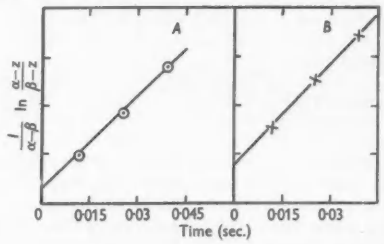

Fig. 3. Plot of $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ versus time. *A*, for data of Fig. 2*A*; *B*, for data of Fig. 2*B*.

term, lz, can be neglected until almost the end of the reaction, and equation (4) on integration then gives

$$l't = \frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z} + \text{constant}.$$

Fig. 3*A*, *B* shows the plot of t versus $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ respectively for the experiments of Fig. 2. The points

fall within experimental error on straight lines of the same slope in the two cases. A similar result was obtained in a duplicate experiment at pH 9·5, and also in a pair of experiments at pH 6·8 (0·05M-phosphate buffer, temp. 16–17°). At the more alkaline pH the reduced haemoglobin is almost completely ionized, and at the more acid pH almost completely unionized, as regards the oxylabile group (pK approx. 8) whose ionization is affected by combination with O_2 or CO, but in both forms the simple equations appear equally applicable. The experiments of Fig. 2 indicate that the reactivity of partially saturated haemoglobin to CO is the same whether the system is in equilibrium or is in a state of change, and thus give no help as to the reason for faster combination of CO with 'new' haemoglobin, referred to in the introduction. No rise in l' is to be seen in the later stages, but this was scarcely to be expected since the reaction was only followed to about 60% of completion. Fig. 4, however, shows the plot of $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ against t for 10 points obtained in a previously published experiment by Bateman & Roughton (1935, fig. 2). In this case the

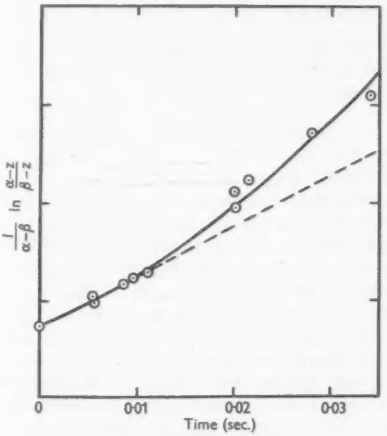

Fig. 4. Plot of $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ versus time for rates of CO combination as given by data of Bateman & Roughton (1935).

rate of combination of CO with haemoglobin was followed to about 85% of completion, by two independent but concordant methods (thermoelectric and photoelectric). The first four points, which just span the first half of the reaction, are seen to fall satisfactorily on a straight line, as in all previous such data. But the latter points clearly lie well above the line and indicate that in the later stages the calculated value of l' tends to increase to 1·4 or so times its initial value. An increase of this order is to be expected on the development of the intermediate compound theory, considered elsewhere by Roughton (1949).

B. *Comparison of haemoglobin solutions and red-cell suspensions.* The previous experimental data used by Roughton (1932) in his theoretical investigation were defective in several respects. (i) The solutions and suspensions were not prepared from the same source of blood, though it is known that the Hb-reaction rates show appreciable scatter between individuals of the same species (Table 1; Roughton, 1932). (ii) The determinations of haemoglobin saturation were made with the reversion spectroscope, which was not only less accurate than the present photoelectric technique, but was, furthermore, not available in the early phases of the process (i.e. saturation 0–30%), wherein the theoretical treatment is most reliable. (iii) Most of the data were obtained on O_2 uptake, which is physiologically of much more interest than CO uptake: from the theoretical point of view, however, the latter process is more satisfactory, in view of its slower rate and absence of appreciable back reaction. We therefore

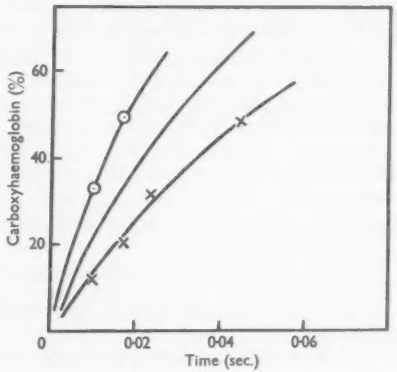

Fig. 5. Rate of combination of CO with reduced Hb solution and with reduced corpuscle suspensions of ram and pregnant ewe blood. Hb solution (ram, ewe), ⊙—⊙; ram cell suspension, ——; pregnant ewe cell suspension, ×—×.

decided to fill in these gaps, by doing several experiments on the rate of CO uptake by haemoglobin solutions and red-cell suspensions, both prepared from the same sheep blood.

The red-cell suspensions were prepared by adding 1 part of blood to 29 parts of Ringer-Locke solution (9 g. NaCl, 0·42 g. KCl, 0·24 g. $CaCl_2$, 0·2 g. $NaHCO_3$/l.). The suspensions were periodically stirred (with minimal air contact) so as to keep them uniform throughout the experiment. For the haemoglobin solutions the blood was laked by adding 14 parts of distilled water, and then 15 parts of double-

strength Ringer-Locke solution. The CO solution was made by shaking Ringer-Locke solution with CO at appropriate pressures, as described above for CO buffer solutions. Four experiments were done altogether, two on ram blood and two on blood from pregnant ewes (kindly supplied by the late Sir Joseph Barcroft).

Fig. 5 shows that the rates of CO uptake by the ram and ewe haemoglobin solution are the same, but considerably faster than the rates by the corresponding cell suspensions, which differ from one another, the rate of the ram cell suspension being about 1·6 times faster than the rate of the ewe cell suspension. This interesting difference was confirmed in other experiments of the series, and is believed to be mainly due to a much lower permeability of the pregnant red-cell membrane. The theoretical interpretation of these results is under investigation by Nicolson & Roughton (1949), and some of their preliminary conclusions have already been published (Legge, Nicolson & Roughton, 1949), though these require modification in view of the recent determinations of the diffusion coefficients of CO and N_2 in 35–40 % (w/v) haemoglobin solutions (Roughton *et al.* 1949).

2. *The rate of dissociation of oxyhaemoglobin in presence of* $Na_2S_2O_4$

A. *Haemoglobin solutions.* Hartridge & Roughton (1923*c*) found that the rate of dissociation of oxyhaemoglobin in presence of $Na_2S_2O_4$ was independent of the concentration of the latter, provided this exceeded a minimal value (approx. 0·1 %). They therefore supposed that a two-stage process takes place, viz:

$$\text{Oxyhaemoglobin} \rightleftharpoons \text{haemoglobin} + \text{dissolved } O_2, \qquad (a)$$

$$O_2 + Na_2S_2O_4 \rightarrow \text{oxidation products of } Na_2S_2O_4, \qquad (b)$$

and that when the limiting $Na_2S_2O_4$ concentration was exceeded, reaction (*b*) proceeded so fast and completely that O_2 is kept negligibly small. Under these circumstances the rate of recombination of dissolved O_2 with haemoglobin in reaction (*a*) would also be negligible, and the overall rate of dissociation of oxyhaemoglobin gave the speed of reaction (*a*) from left to right without appreciable back reaction. The rate of dissociation of oxyhaemoglobin, so observed, was found to follow a unimolecular course from 80 % O_2Hb downwards. Millikan's more accurate work led to the same conclusion, and his experiments on the rate of dissociation of oxyhaemoglobin and oxyhaemocyanin in presence of $Na_2S_2O_4$ gave added support to the original mechanism which has for some time been generally accepted. There are, however, still some points in regard to the speed and order of reaction (*b*) which need to be settled if the whole process is fully to be understood.

Fig. 6 shows the results of one of two concordant experiments at pH approx. 10, 0·1 % $Na_2S_2O_4$, on the rate of dissociation when the haemoglobin was initially (i) 99–100 % saturated, (ii) 50–60 % saturated. The points in the latter case fall, within experimental error, on the same curve as in the former

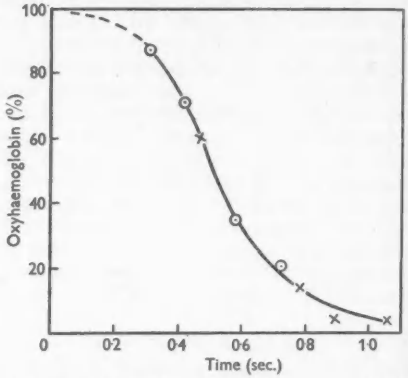

Fig. 6. Rate of dissociation of O_2Hb in presence of $Na_2S_2O_4$ at pH 10, temp. 11°. ⊙ = 100 % O_2Hb at start; × = 60 % O_2Hb at start.

case. The curve is logarithmic between 70 and 0 % O_2Hb, and in this range the kinetics of the reaction thus conform to the equation

$$\frac{-d[O_2Hb]}{dt} = k[O_2Hb].$$

Between 100 and 80 % O_2Hb the curves of this paper show a lag period, as do also Millikan's curves (1932), when closely inspected. This lag has hitherto been attributed to reaction with the $Na_2S_2O_4$ of the excess of dissolved O_2, which is initially present just after mixture (i.e. at about 75 mm. pO_2, where pO_2 = partial pressure of O_2) and has to be reduced to a low value (e.g. about 10 mm. pO_2) before reaction (*a*) can occur to an appreciable degree. The lag period, however, continues further than would be expected if this were the whole explanation. This point may be illustrated by considering in more detail the four points in the experiment of Fig. 6, in which the O_2Hb started by being 99–100 % saturated. The actual numerical data of y and t are shown in Table 1,

Table 1. *Determination of velocity constant for rate of dissociation of oxyhaemoglobin over various time intervals*

Percentage saturation (y)	Time in sec. (t)	k
100	0	
		0·42
87	0·31	
		1·85
71	0·42	
		4·4
35	0·58	
		3·9
20·4	0·72	

together with the calculated values of k over various time ranges. The value of k is seen to rise to a plateau, but does not reach it until y has fallen to 70 %. Consider the range between $y = 87$ % and $y = 71$ %. At $y = 87$ % the equilibrium pO_2 at the temperature of the experiment, 11°, would be 2·7 mm., and at $y = 70$ % it would be 2·0 mm. The maximum amount of physically dissolved O_2 which the $Na_2S_2O_4$ would have to remove over this range would only be $\frac{2{\cdot}6 - 2{\cdot}0}{760} \times 0{\cdot}037$ (= solubility coefficient of O_2 in water) = 0·000034 ml./ml. The combined O_2 which is removed on the other hand amounts to

$$\frac{87 - 71}{100} \times 0{\cdot}003 \text{ (= gas-combining capacity of Hb sol.)}$$
$$= 0{\cdot}00048 \text{ ml./ml.}$$

As the latter figure is 14 times greater than the former, it seems impossible that the low value of k in the range 87–71 % O_2Hb should be appreciably due to the dissolved O_2 effect. It must, in fact, be in the main due to a genuine kinetic feature of the dissociation of O_2Hb, as is indeed to be expected on the theory put forward by Roughton (1949). This feature had hitherto escaped notice, partly owing to the paucity of dissociation rate data above $y = 80$ % and partly through being obscured by the dissolved O_2 effect, the importance of which is undoubted between $y = 100$ % and $y = 95$ % (about), but fades out very rapidly when y falls below 95 %.

Fig. 7 shows the results of one of two concordant experiments at pH 6·8. In these cases, owing to the greater speed of the reaction, there are not sufficient data in the upper range for a decision as to an initial lag period. Both experiments showed a rather faster initial rate of dissociation in the case of the partially saturated haemoglobin than would be expected from the curve for the fully saturated haemoglobin. The effect is, however, overshadowed by a more remarkable finding, namely that the dissociation appears to proceed in two phases, a rapid one from 100 to about 30 % O_2Hb, and then a slow one from 30 to 0 % O_2Hb. This latter phase takes several seconds to reach completion, as was shown by following the progress of the change when the running fluid was suddenly stopped. Human haemoglobin at pH 6·0–7·4 (Fig. 2; Millikan, 1936) shows a similar diphasic effect, which is brought out clearly when the logarithm of O_2Hb concentration is plotted against time. The discrepancy in the rates of the two phases is, however, not so great as in Fig. 7. Perhaps this was why Millikan did not notice or comment on the effect. Later in this section the matter is investigated and discussed further.

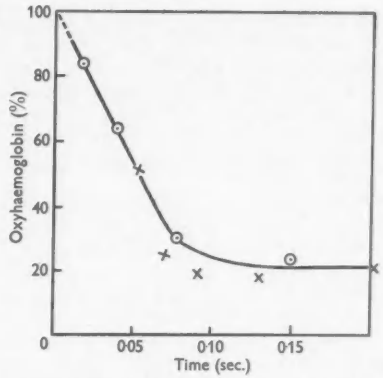

Fig. 7. Rate of dissociation of O_2Hb in presence of $Na_2S_2O_4$ at pH 6·8, temp. 15·8°. ⊙ = 100 % O_2Hb at start; × = 51 % O_2Hb at start.

B. *Comparison of haemoglobin solutions and red-cell suspensions.* Roughton (1932) found that the rate of dissociation of oxyhaemoglobin in sheep red-cell suspensions was about one-quarter of the rate in homogeneous solution. A similar ratio is suggested by Dirken & Mook's (1931) data on ox haemoglobin, though Millikan's results (1932) for human haemoglobin indicate a rather lower figure. In nearly all this earlier work, however, the pH of the interior of the red cells was probably not the same as that of the haemoglobin solutions, and part of the observed difference in the dissociation rates might be due to difference of pH, which has a large effect *per se*.

We have tried to obviate this difficulty by working at the isoelectric point of haemoglobin (pH approx. 6·8), for here there is practically no difference in pH between the interior of the red cells and the surrounding liquid.

Table 2. *Comparison of rate of dissociation of oxyhaemoglobin in red-cell suspensions and haemoglobin solutions (ram)*

Ram no.	pH	Temp. (°)	Half time of dissociation (sec.) (*a*) Hb solution	(*b*) Cell suspension	(*a*)/(*b*)
1	6·8	15	0·03	0·14	0·21
2	6·8	13·5	0·03	0·16	0·19
	7·1	13·5	0·05	0·21	0·23
3	6·8	14	0·03	0·12	0·25
	7·1	14	0·03	0·17	0·18

The red-cell suspension, the haemoglobin and the $Na_2S_2O_4$ were therefore all made up in Ringer-Locke solution buffered with a bicarbonate-CO_2 mixture of pH 6·8–7·1. The procedure was thus the same as in section 1, save that the $NaHCO_3$ concentration in the Ringer-Locke solution was increased from 0·8 g./l. to 4·1 g./l., and all the solutions were finally equilibrated with CO_2 at pressures of 150–300 mm. Hg. The final concentrations in the mixed fluid were 1 g. $Na_2S_2O_4$ and 1 part of blood in 60.

Table 2 summarizes the results of comparisons carried out with blood from three different rams. The mean ratio, at pH 6·8, for the rate in cells to the rate in solution is 0·22, and is about the same at pH 7·1, thus confirming the results of the earlier experiments. The mathematical interpretation of the present data is also under investigation by Nicolson & Roughton (1949).

C. *Further study of the apparent diphasic dissociation of oxyhaemoglobin at neutral* pH. This observation aroused our interest for several reasons. (i) It might have some fresh bearing on the nature of the reaction between oxygen and haemoglobin, and in particular might provide new evidence that one of the four haem groups in the molecule behaves differently from the other three. (ii) It might be related to Roughton's (1934*d*) unexplained observation that when dilute sheep oxyhaemoglobin, between pH 6 and 7, is suddenly mixed with O_2-free water, the oxyhaemoglobin dissociates in two stages, about two-thirds of the oxygen coming off in about 0·5 sec. and the remainder over a period of several seconds. (iii) In the circulating blood *in vivo* it is often observed that the venous blood contains appreciable reserves of oxygen even when the whole body, or particular organs of it, are in a state of severe oxygen want. It seemed possible that a slow final phase in the rate of oxyhaemoglobin dissociation might be a factor concerned in this effect.

We therefore carried out some thirty experiments altogether in the hope of elucidating the matter further. Unhappily the results were irregular and it was only in the latter weeks of our collaboration that we got satisfactory clues as to the sources of the varying effects.

In fifteen experiments at pH 6·8 (0·05 M-phosphate buffer, temp. 9–19°) a break was found in every case, but the percentage saturation at which the break occurred (referred to as the 'plateau') varied from 3 to 30 % O_2Hb. Three to five tests were also made at each of the following pH values: 6·2, 7·1, 7·4 and 7·7 (all in phosphate buffer) and at pH 6·8 and 7·1 in CO_2-bicarbonate buffer. In the three tests at pH 6·2, the plateau occurred at a much lower percentage O_2Hb than with the same haemoglobin solution at pH 6·8, but at the other pH values there was no marked difference from the results at pH 6·8.

In three out of four experiments on the cell suspensions (in CO_2-bicarbonate buffer, pH 6·8–7·1), no distinct plateau was seen at all and in the fourth one, only a rather low 'plateau' at about 9 % O_2Hb. There thus appeared to be a genuine difference between the haemoglobin solutions and the corpuscle suspensions, and since $Na_2S_2O_4$, and its products of oxidation, are not believed to permeate the red-cell membrane, or at most only very slowly, it seemed that the diphasic effect might be associated with some action of $Na_2S_2O_4$ or its oxidation products on the haemoglobin. In a control experiment in which the $Na_2S_2O_4$ concentration was varied two-fold, the diphasic course of the reaction was unaffected: this suggests that the products of reaction of $Na_2S_2O_4$ with O_2, rather than the $Na_2S_2O_4$ itself, are responsible since their concentration (under constant haemoglobin conditions) would hardly be altered when the $Na_2S_2O_4$ concentration is halved or doubled.

Further support for this view was obtained along the following lines. In the majority of the later tests the mixture of O_2Hb and $Na_2S_2O_4$, which had run through the apparatus, was allowed to stand for 15 sec. and then slowly siphoned back through the observation tube, so as to serve as the source of reduced haemoglobin for calibration purposes at each observation point. This standard may be referred to as reduced haemoglobin *A*. Two other forms of reduced haemoglobin standard were also used on various occasions. In both cases a solution of completely reduced haemoglobin (prepared as described in the section on experimental methods) was mixed in the reaction apparatus with the $Na_2S_2O_4$ solution and either collected and similarly siphoned back through the observation tube (= reduced haemoglobin *B*) or readings were taken on the mixed fluid as it travelled down the observation tube before collection (= reduced haemoglobin *C*). In certain of the later experiments* the reading given by reduced haemoglobin *A* differed appreciably (in the sense of appearing to be more reduced) from that given by reduced haemoglobin *B* or *C*, and in such cases a high 'plateau', of the order of 20 % O_2Hb was seen. On the other hand, in five experiments in which no appreciable difference in reading between reduced haemoglobin *A* and *B* (and/or *C*) was observed, the 'plateau' was always low, i.e. not more than 10 % O_2Hb. Our general conclusion was thus that in certain cases the oxidation products of $Na_2S_2O_4$ produced abnormal haemoglobin pigments, and when this happened a much more pronounced, apparent 'plateau' was found in the rate of dissociation of the oxyhaemoglobin.

* In one case reduced haemoglobin standard *A* was also made from blood dissolved in oxygenated water (pO_2 = 730 mm. Hg) as well as from the usual blood dissolved in aerated water, (pO_2 = 150 mm. Hg). The oxygenated Hb on mixing with the $Na_2S_2O_4$ gave a standard which appeared, photocolorimetrically, to be distinctly more 'reduced' than the aerated Hb, when similarly treated.

This view was tested further by collecting the reaction mixture as it left the observation tube in a test tube half filled with CO-saturated buffer containing $Na_2S_2O_4$. When the test tube was full, the solution, now 1 in 120 with respect to the original blood, was examined immediately with a hand spectroscope or a Hartridge reversion spectroscope. The light path could be made to traverse up to 15 cm. of liquid. When O_2Hb (partial pressure of $O_2 = 0{\cdot}2$–$1{\cdot}0$ atm.) and buffered $Na_2S_2O_4$ were allowed to react and were examined in this way, a faint but definite band was visible at 628 mμ. in one experiment. No such band was observed when reduced haemoglobin and $Na_2S_2O_4$ were allowed to react and were similarly examined. The 'plateau' in this experiment was at about 18 % O_2Hb, i.e. in the higher range.

The blood used in this experiment was stored overnight in the refrigerator and the experiment repeated next day under as closely similar conditions as possible. In this case the band in the red was less pronounced, and the 'plateau' was found to have dropped to about 3 % O_2Hb. As to the cause of the difference of behaviour on the two successive days we have at present no clue, but the correlation of the pronounced 'plateau' with the appearance of the band in the red is striking. The conditions under which the latter was obtained are consistent with the supposition that CO-choleglobin (Legge & Lemberg, 1941) or perhaps CO-cruoralbin (Holden, 1943) had appeared.

For the present purpose it is unimportant whether choleglobin or cruoralbin is formed. Although the band observed was that of a ferrous CO compound (the CO being used to stop further coupled oxidation and to form a derivative with a sharp banded spectrum), the ferrous forms of both the above compounds have absorption bands in the 620–630 mμ. region in the absence of CO. The presence of either, in sufficient amount, could invalidate our interpretation of the galvanometer deflexion in terms of only oxy- and reduced haemoglobin. In view of the arbitrary way in which the galvanometer was adjusted to zero by movement of the red-green filter it is not possible to state, on the basis of the published data on the absorption of choleglobin or cruoralbin, what concentration of these compounds would be required in order to simulate a given oxyhaemoglobin-reduced haemoglobin mixture in the apparatus.

Additional evidence that part of the 'diphasic' course of the reduction may be accounted for by the presence of other pigments is provided by a few experiments which were carried out for us by Dr B. Chance with his refined photoelectronic rapid reaction apparatus. In these experiments monochromatic light of wavelength 578 mμ. was used, the percentage reduction being obtained by the difference in absorption of oxy- and reduced haemoglobin at this wavelength. The reduction appeared to be a first order reaction all the way, no significant second phase being detected.

This tentative explanation of the 'diphasic' reduction of oxyhaemoglobin does not, however, account for all the aspects of the phenomenon. The fact that a slow decline in the apparent concentration of oxyhaemoglobin is observed during the second phase of the reduction, is inconsistent with the supposition that the concentration of choleglobins is increasing at this period in the course of the reactions, and suggests that a fourth compound, in addition to oxyhaemoglobin, reduced haemoglobin and choleglobin (or cruoralbin), may be present. This might be a precursor of the choleglobin observed spectroscopically 2–3 sec. after mixing. Further investigation of the problem, however, will have to await an apparatus such as the one constructed by Chance (1947), which is able to record the absorption at several wavelengths simultaneously.

Although the matter has not yet been fully worked out, we do feel it reasonable to conclude that a large part of the apparent 'plateau' when the latter is high, is due to the appearance of abnormal pigments, rather than to a genuine diphasic dissociation of oxyhaemoglobin. We cannot, however, at present exclude the possibility that the latter may exist in a minor degree, but to be sure of this it would be most desirable to use gasometric as well as spectroscopic methods of detection. If, and only if, such combined tests turned out positive, would it be worth while to consider further the bearing of the 'plateau' upon the three questions, listed as (i), (ii), and (iii) at the beginning of the present section 2C. As regards the apparently faster initial rate of dissociation of the partially saturated haemoglobin, shown in Fig. 7, it may be largely due to the presence of a smaller proportion of oxidized $Na_2S_2O_4$ products than in the case of the fully saturated haemoglobin.

Two other possible explanations of the 'plateau' we believe have already been ruled out. (i) Hydrogen peroxide is known to be formed during the oxidation of $Na_2S_2O_4$ and this might be decomposed by the blood catalase and the oxygen so formed reoxygenate the haemoglobin, thus setting up a temporary cyclic process. In a control experiment in which sodium azide was added to give a final concentration of 0·001 M-sodium azide (so as to inhibit completely the catalase of the blood solution) no difference in the diphasic course of the reaction was found, the plateau remaining at approx. 18 % O_2Hb. (ii) That the $Na_2S_2O_4$ may only react slowly with dissolved O_2, at the very low partial pressures of the latter which must exist during the last stages of dissociation of O_2Hb. If so, the red-cell suspensions should also be similarly affected, which they are not: furthermore, the speed of the slow phase should be dependent on the concentration of $Na_2S_2O_4$, which has already been shown not to be the case.

In conclusion it may be noted that dilute solutions of haemoglobin and of $Na_2S_2O_4$ are both much more labile at pH 7 and below (i.e. the range in which these effects appear) than at alkaline pH. Although $Na_2S_2O_4$ has proved of great service in studying the kinetics of dissociation of oxygen-carrying pigments, it is a great pity that no satisfactory alternative oxygen absorbent has been discovered for this purpose, particularly in the neutral pH range.

SUMMARY

1. The rate of combination of X with haemoglobin (Hb), where X = oxygen or carbon monoxide, has hitherto been expressed by the equation $d[XHb]/dt = k'[X][Hb] - k[XHb]$, which has also been applied to the dissociation of X from haemoglobin. In the later stages of combination, however, it is now found that the calculated value of k' tends to rise appreciably, whereas in the early stages of dissociation of oxyhaemoglobin (O_2Hb), k tends to be much lower than the plateau value which it attains after the dissociation is about one-third completed. These discrepancies are in line with expectations based on the intermediate compound theory.

2. In all previous kinetic work the haemoglobin at time zero has been either completely in the form XHb, or in the form Hb. Theoretically discrepancies might occur if the haemoglobin was partially saturated at the outset of the reaction. Experimental tests, however, gave negative results in the case of combination of carbon monoxide with Hb at pH 6·8 and 10·0, and also in the dissociation of O_2Hb in presence of $Na_2S_2O_4$ at pH 10·0, but at pH 6·8 a slight positive effect was observed with the latter reaction.

3. At pH 6·8, and at neighbouring pH, the dissociation of O_2Hb in presence of sodium dithionite ($Na_2S_2O_4$) seems to occur in two phases, a fast one from 100% O_2Hb to 20 ± 10% O_2Hb, followed by a slow final one taking several seconds. Controls showed that a large part of this effect is probably due to secondary effects of the oxidation products of $Na_2S_2O_4$ upon Hb, leading to formation of choleglobin and related products, the presence of which might well interfere with the estimation of the O_2Hb/Hb ratios by the Millikan photocolorimetric method used in this paper.

4. New determinations are given of the comparative rates of combination of carbon monoxide with sheep haemoglobin in solution and in red-cell suspensions. Similar experiments on the rates of dissociation of O_2Hb in solution and in the red cell are also given.

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The Thiocyanate Space and Iodide Space in the Thyroid Gland

By J. F. McCLENDON, W. C. FOSTER and E. REED
The Hahnemann Medical College, Philadelphia and the Experimental Farm R. 1, Box 383, Norristown, Pennsylvania

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Both thiocyanate and iodide have marked effects on the thyroid and both ions have been used to measure the distribution of available water in the body. It is clear that iodide space may not always be a measure of water distribution because the thyroid cell is able to concentrate iodide. The present paper is concerned with the iodide space and the thiocyanate space in the thyroid of the dog. We define thiocyanate space and iodide space as the ratio of the concentration of these substances in protein-free organs to that in protein-free blood plasma. After we had finished the determinations reported here we discovered the paper by Baumann & Metzger (1949) dealing with the same problem, but since these authors used an entirely different technique, and ours are the only experiments in which the volume of the thyroid was determined, we felt that our data were worth publishing.

EXPERIMENTAL

Method of estimation of thiocyanate. It appeared undesirable to hydrolyse tissue with ethanolic KOH according to Brodie & Friedman (1937). We began working with the thiocyanate method of Crandall & Anderson (1934) using trichloroacetic acid to precipitate the proteins. This method depends on measuring the colour of ferric thiocyanate which is red. In determining thiocyanate in blood plasma we were disturbed by the admixture of a green colour. Since ferrous thiocyanate is green, we supposed that the green colour was due to the reduction of ferric iron to ferrous iron by reducing substances of the tissues. We reduced the error due to ferrous iron by increasing the concentration of ferric iron ten times and working rapidly. Furthermore, in order to eliminate the possibility of the green colour interfering with the estimation of ferric thiocyanate, we estimated the latter by means of a Coleman photoelectric spectrophotometer set at 5000 A. Graphs were made, plotting the log. of the reciprocal of transmission against mg. of Na thiocyanate in various dilutions of a standard solution. This graph was a straight line and was used in determining the thiocyanate concentration of the experimental samples. By this method we determined the thiocyanate space in the dog thyroid gland to average 0·45 (45%).

Animals. Dogs were selected with no externally visible thyroids, but one had a noticeable enlargement of the thyroid on thyroidectomy. They were divided into two groups of thirteen dogs each. Group 1: each dog was injected in the femoral vein with 30 ml. of 1% (w/v) NaI (except where specified otherwise, Table 1). After 2–3 hr. (Table 1), 5 ml. of blood were drawn from the femoral vein under pentothal anaesthesia into a centrifuge tube containing 3 mg. of K oxalate as a film on its walls, and the thyroid gland removed. Group 2: each dog was injected in the femoral vein with 30 ml. of a solution of 1% NaI to which had been added 1 g. of NaCNS/100 ml. Blood and thyroid were removed after 3 hr. as with group 1.

Thiocyanate estimation in the protein-free filtrate of oxalated blood plasma. Plasma (1 ml.) was delivered into a 15 ml. centrifuge tube and diluted to 5 ml. with distilled water. 20% (w/v) Trichloroacetic acid (5 ml.) was then added and the two solutions thoroughly mixed to ensure complete precipitation. After standing for 10 min. the precipitate was centrifuged for 5 min. and the protein-free supernatant fluid transferred directly to the spectrophotometer cell with addition of 0·5 ml. of conc. ferric nitrate reagent (50 g. $Fe(NO_3)_3.6H_2O$ and 100 ml. conc. HNO_3 in 100 ml. water). The reading was made rapidly and compared with the graph.

Handling of the thyroid gland. The thyroid gland was dissected out immediately after the dog was killed and the volume was determined by placing the gland in a 10 ml. graduated cylinder containing exactly 1 ml. of distilled water, and noting the change in volume. The gland was then removed from the water, transferred to a watch glass and frozen solid in the ice-making compartment of the electric refrigerator. The frozen tissue was quickly sliced, finely diced with a sharp scalpel and crushed in a supercooled diamond mortar and placed in a Wisconsin homogenizer. After homogenizing for 7 min. the tissue was transferred to a centrifuge tube and the homogenizer washed with small amounts of distilled water which were added to the tissue until a total volume of 5 ml. had been reached. 20% Trichloroacetic acid (5 ml.) was then added and the procedure followed as with plasma.

Iodine determination. The protein-free supernatant from 1 ml. of plasma was evaporated and fused in a nickel crucible with two pellets of NaOH. 10 ml. of an azide-carbonate solution (500 mg. NaN_3 and 500 mg. Na_2CO_3 in 100 ml. twice distilled water) was added and the solution brought to boiling and transferred to the microdistilling flask. The iodine was then determined by the method of McClendon & Bratton (1938). The iodine in the protein-free centrifugate of the thyroid gland was determined in the same manner.

RESULTS AND DISCUSSION

The iodide space in the thyroid gland of dogs injected with sodium thiocyanate (Table 2) was as large as that in dogs not so injected (Table 1).

It is evident that the iodide space, averaging 3·7–4·26 (grand average 3·98) times the total volume

Table 1. *Iodide space in thyroid gland of dogs injected with sodium iodide solution*

Dog wt. (kg.)	Vol. of NaI (1% w/v) injected (ml.)	Interval between injection and taking of samples (hr.)	(min.)	Thyroid vol. (ml.)	Iodide space
8·0	30	2	10	0·8	0·66
16·2	30	2	05	2·6	1·70
15·6	30	2	10	2·2	2·44
9·4	30	2	05	0·9	2·10
7·8	30	2	10	1·5	2·20
11·5	30	2	30	0·6	2·30
22·0	30	2	30	2·4	2·40
7·1	30	1	45	1·2	2·44
15·0	20	3	30	0·9	5·26
8·0	30	2	30	0·4	5·40
6·2	30	2	45	1·0	5·60
8·5	10	3	15	1·0	6·00
24·0	20	2	40	1·3	10·80
				Av.	**3·70**

of the thyroid gland cannot be a measure of the available water in the gland. The thyroid may concentrate iodide just as the brown seaweed does. On the other hand, thiocyanate seems not to interfere with the absorption of iodide by the thyroid gland when equal quantities of their sodium salts are injected intravenously and measured after 2–3 hr. Perhaps if much larger quantities of thiocyanate or much smaller quantities of iodide had been injected, or the samples taken earlier than 2 hr. after the injection, some effect on the absorption of iodide might have been obtained. We used large quantities of iodide to make the analytical work more accurate. Most of the iodine in the thyroid is bound to proteins and the volumes of these dog thyroid glands were (with one exception) smaller than the blood samples we usually take for iodine analysis. The iodide space in the thyroid gland was more than nine times as large as the thiocyanate space and thus the conclusions of various workers that the thyroid selectively absorbs iodide is confirmed.

Table 2. *Iodide space in thyroid of dogs after injection of sodium iodide and sodium thiocyanate*

(Samples taken 3 hr. after injection of NaI (300 mg.) and NaCNS (300 mg.).)

Dog wt. (kg.)	Thyroid vol. (ml.)	Iodide space
7·0	0·7	0·64
15·0	9·0	0·73
6·0	0·4	0·78
7·7	0·7	0·94
10·8	0·6	0·94
6·5	0·5	1·79
6·4	0·8	3·40
8·2	1·1	4·94
7·0	0·7	5·45
8·7	0·4	7·05
14·0	1·5	8·57
8·4	0·4	9·28
5·5	0·6	10·81
	Av.	**4·26**

The variation in individual values of the 'iodide space' in Tables 1 and 2, added to the fact that it is usually greater than unity indicates that it is not a measure of the available water in the thyroid, but a measure of the concentrating activity of the thyroid cells. Until we know more of the concentrating mechanism it would seem premature to discuss this point. Since the literature on this subject is covered by Leblond (1948), and Stanley & Astwood (1948), we need only refer to a more recent paper. The generalization of Baumann & Metzger (1949) that all elements of Group VII of the Periodic Table except iodine cause goitre is not in harmony with all the literature. In fact, Shrewsbury, Hatfield, Doyle & Andrews (1944) conclude that fluoride prevents goitre, but they did not make iodine analyses.

SUMMARY

1. The thiocyanate space in the thyroid gland of dogs averaged 0·45 (45%). The iodide space in the thyroid gland of dogs not receiving thiocyanate averaged 3·7 (370%) and of those receiving thiocyanate 4·26 (426%) 2–3 hr. after the injections. In other words, these analyses do not support the idea that thiocyanate interferes with the absorption of iodide by the thyroid gland.

2. In the analysis of thiocyanate in living tissue, the formation of ferrous thiocyanate is to be avoided.

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Biochemistry of the Wood-rotting Fungi

6. VOLATILE METABOLIC PRODUCTS OF SPECIES OF *ENDOCONIDIOPHORA*

By J. H. BIRKINSHAW and E. N. MORGAN
Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received* 30 *December* 1949)

The substances responsible for the characteristic odours of the fungi *Lentinus lepideus* Fr. and *Trametes suaveolens* (Linn.) Fr. have been isolated and characterized (Birkinshaw & Findlay, 1940; Birkinshaw, Bracken & Findlay, 1944). The odours are due mainly to aromatic esters, amongst which the methyl esters of *p*-methoxycinnamic, cinnamic, and anisic acids were identified in the products of *Lentinus lepideus*, and methyl anisate in the products of *Trametes suaveolens*. The latter fungus also produced anisaldehyde which was a contributor to the odour.

This work has now been extended to an investigation of the odoriferous products of species of *Endoconidiophora*. Some of the products isolated were briefly reported (Morgan, 1948) whilst the investigation was in progress; a complete account of this work forms the subject of the present communication. *Endoconidiophora* is a genus established by Münch to include the species of *Ceratostomella* which produce secondary spores in an endogenous manner (i.e. from within the terminal cell and not by abscission of the ends of the hyphae). It includes a number of species important as a cause of 'blue stain' or 'sap stain' in logs and sawn timber. *Endoconidiophora coerulescens* is an important cause of staining in sap wood in Europe, and according to Davidson (1944) *E. virescens* is one of the principal fungi causing stain in hardwoods in the southern United States. *E. fimbriata* is said to be of little importance as a stainer of timber.

The bluish tinge produced by these fungi is not due to a pigment, but is an optical effect caused by the scattering of light rays striking the very fine dark hyphae. The bluish colour of diluted milk is produced in a similar way. Whilst sap stain has little effect on the mechanical properties of the commoner European soft woods, it may be more serious in soft tropical woods.

When some *Endoconidiophora* species are grown under laboratory conditions a pleasant fruity odour is evolved. Davidson (1944) refers to the banana-oil (amyl acetate) odour as a characteristic of *E. coerulescens*. He describes the odour of *E. virescens* as musty and penetrating. *E. coerulescens* was selected, from the species made available to us, as yielding the strongest odour, and was cultured in quantity on a liquid malt medium.

After a few days the odour permeated the incubator room. After harvesting, the fungal mycelium and culture fluid were subjected to steam distillation. The steam-volatile products were then distilled. Two main fractions were obtained. The lower boiling fraction was found to consist of *iso*butyl acetate ($HCMe_2.CH_2.O.CO.CH_3$); the higher fraction contained methylheptenone (2-methylhept-2-en-6-one) and a mixture of methylheptenols (2-methylhept-2-en-6-ol) in which the dextrorotatory isomer and the racemic form were detected and characterized as allophanates. Since (+)-methylheptenol has been shown by Levene & Haller (1929) to be configurationally related to (+)-lactic acid, which is now denoted as L-lactic acid, the two methylheptenols isolated are correctly described as L-2-methylhept-2-en-6-ol and the corresponding DL-compound.

Two other species of *Endoconidiophora* were readily available and were examined by way of comparison. *E. virescens*, which gave a less pronounced odour than *E. coerulescens*, produced methylheptenone and L- and DL-methylheptenol, but no *iso*butyl acetate could be detected. None of these products was isolated from *E. fimbriata*, which

also produced practically no odour. The amyl acetate odour referred to by Davidson (1944) as characteristic of *E. coerulescens* is no doubt that of *iso*butyl acetate. The fact that this odour is not produced by *E. virescens*, which is one of the reasons advanced by Davidson (1944) for separating *E. virescens* from *E. coerulescens* as a distinct species, agrees with our failure to find *iso*butyl acetate in the volatile metabolic products of *E. virescens*.

The occurrence of *iso*butyl acetate in nature does not appear to have been previously recorded, but the alcoholic component, *iso*butanol, occurs in fusel oils and certain essential oils. It is found in Java citronella oil together with methylheptenone (Bohnsack, 1943). It occurs, esterified with angelic acid and with *iso*butyric acid, in Roman chamomile oil (Köbig, 1879).

Acetic acid is probably formed in traces by a number of fungi since many have been shown to yield small amounts of volatile acid, and one of its esters, ethyl acetate, is a product of the mould *Penicillium digitatum* (Birkinshaw, Charles & Raistrick, 1931).

Methylheptenone is a constituent of various natural essential oils such as lemongrass oil, palmarosa oil, linaloe oil, cacao oil and verbena oil. An optically active form of methylheptenol has been recorded in linaloe oil.

Methylheptenone is readily convertible by chemical reduction to the DL-methylheptenol and it seems likely that the fungus may achieve a similar reduction and that, under biological conditions, the two possible stereoisomerides might be formed in unequal amounts. This is in accordance with the detection of L-methylheptenol in addition to the DL-compound as a product of *Endoconidiophora* species. The methylheptenol isolated from linaloe oil had a small negative rotation ($[\alpha]_D - 1{\cdot}57°$: Schimmel & Co., 1908); this indicates a slight preponderance of the D- over the L-isomeride, since the value is much lower than that for the pure enantiomorphs.

The possibility that the synthesis of the alcohol by *Endoconidiophora* species precedes that of the ketone must not be overlooked, but if that were the case it might be expected that one stereoisomeride would be formed to the exclusion of the other.

It is of some interest to note that Neuberg & Lewite (1918) attempted the phytochemical reduction of various ketones by adding them to a yeast fermentation, and found that, of the ketones examined, only methylheptenone was reduced in these conditions. In two experiments apparently differing only in the time of incubation the optical rotation of the product was of opposite sign in the two cases. After 2 months' incubation the methylheptenol had $[\alpha] - 6{\cdot}50°$, after 3 months' incubation, $[\alpha] + 2{\cdot}06°$. Here again the products must have been mixtures of the D- and L-methylheptenols in unequal amounts. In later work (Neuberg & Nord, 1919; Akamatsu, 1923) various aliphatic, aromatic and cyclic ketones were shown to be susceptible to phytochemical reduction by yeast, with production of optically active secondary alcohols.

The association of *iso*butanol (or its esters), methylheptenone and methylheptenol as metabolic products of both green plants and of fungi and their obvious chemical relationship suggests that the mechanism of the synthesis may be similar in the two cases.

EXPERIMENTAL

Cultures. The cultures were obtained through the courtesy of Dr W. P. K. Findlay from the Forest Products Research Laboratory, Princes Risborough (F.P.R.L.).

1. *E. coerulescens* Münch—F.P.R.L. No. S87—isolated by Rennerfelt in Sweden.

2. *E. virescens* Davidson—F.P.R.L. No. S81—U.S. Department of Agriculture, Division of Forest Pathology, No. 94255.

3. *E. fimbriata* (Ell. & Hals.) Davidson—F.P.R.L. No. S90—isolated by Davidson from brown-stained beech, received from Centraalbureau voor Schimmelcultures.

Preparation of inoculum. The cultures for inoculation were grown on a sterile Norway spruce sawdust medium containing 10% of an accelerator consisting of 50% maize meal, 30% bone meal and 20% A.R. starch (Badcock, 1941). The sawdust (33·3 g.) and accelerator (3·7 g.) were well mixed and moistened with distilled water (75 ml.). The moist mixture was lightly packed into large boiling tubes which were sterilized by autoclaving.

Incubation of the inoculated sawdust medium at 24° resulted in a fairly rapid development of the fungus throughout the medium. The progress of the infection could be followed visibly in the cases of *E. coerulescens* and *E. virescens* owing to the very dark colour of the developing hyphae. For inoculation of the culture medium about 1 g. of the infected sawdust was added to each flask.

Medium and conditions of culture. The culture solution employed consisted of 4% (w/v) of Vimaltol in distilled water (Vimaltol is a proprietary malt extract with addition of vitamins A, B_1, D, niacin and iron). The medium was distributed in conical flasks of 1 l. capacity, 350 ml. to each flask. The flasks were plugged with cotton wool, autoclaved at 110°, inoculated with the infected sawdust and incubated at 24°.

PRODUCTS FROM *ENDOCONIDIOPHORA COERULESCENS*

After 4–5 days a complete mycelium extended over the surface of the medium and a pleasant ester-like odour was observed which in the later stages of growth permeated the incubator room. After 15 days' incubation, when the fungus was harvested, the fragile mycelium had a mottled steel-grey to black upper surface with a black reverse. The total contents of the flasks were then subjected to steam distillation. The distillate contained colourless globules of oil floating on the surface of the water. The oil was collected by extraction with ether and careful removal of the solvent. Since the culture fluid inoculated with another species (*E. fimbriata*) gave only a trace of steam-volatile material, the oil obtained with *E. coerulescens* cannot have been pro-

duced from the medium alone. The extracted oil from ninety-two flasks was subjected to distillation and gave the following fractions: fraction I, 0·65 g., b.p. 110–120°/764 mm.; fraction II, 4·48 g., b.p. 173–176°/764 mm., $n_D^{20°}$ 1·443; undistilled residue 0·58 g.

Treatment of fraction I

Fraction I was a colourless mobile oil with a pleasant ester-like smell. The oil (0·3865 g.) was hydrolysed by refluxing on a boiling-water bath with 10 ml. of N-NaOH for 2 hr. The reaction mixture was extracted with ether. The residue on removal of the ether was an oil (0·122 g.) with an odour reminiscent of *iso*butanol. Its identity was confirmed by heating it with 3:5-dinitrobenzoyl chloride (0·250 g.) in benzene (5 ml.) containing a few drops of pyridine for 0·5 hr. on a water bath. The cooled reaction mixture was diluted with ether, washed with dilute NaOH, dilute HCl and water, and the solvent was removed *in vacuo*. The residue (0·152 g.) after recrystallization from light petroleum gave colourless needles (95 mg.), m.p. 86°, undepressed on admixture with the authentic 3:5-dinitrobenzoate, m.p. 86–87°, of *iso*butanol. (Found: C, 49·1; H, 4·5; N, 10·2. Calc. for $C_{11}H_{12}O_6N_2$: C, 49·3; H, 4·5; N, 10·4%.) The 3:5-dinitrobenzoates of *n*-butanol and of *sec.*-butanol have m.p.'s 64° and 75° respectively. The m.p. of the mixture of the dinitrobenzoate of the latter alcohol with that of the metabolic product showed marked depression.

The volatile acid present as the Na salt in the ether-extracted alkaline layer was obtained after acidification with N-H_2SO_4 by repeated distillation to low volume with addition of successive quantities of distilled water. The total volatile acid in the aqueous distillate required 25·75 ml. of 0·1 N-NaOH for neutralization to phenolphthalein. The concentrated aqueous solution of the Na salt (4 ml.) was refluxed with ethanol (8 ml.) and *p*-bromophenacyl bromide (0·70 g.) for 75 min. The reaction mixture deposited on cooling a small amount of unchanged reagent. This was removed by filtration, and the filtrate was diluted with water to give crystals, which after recrystallization from light petroleum had m.p. 84–85° (0·10 g.) and showed no depression on admixture with authentic *p*-bromophenacyl acetate of m.p. 85°. (Found: C, 46·7; H, 3·3; Br, 31·0. Calc. for $C_{10}H_9O_3Br$; C, 46·7; H, 3·5, Br, 31·1%.)

Fraction I therefore consisted substantially of *iso*butyl acetate. Further confirmation was obtained by determination of the apparent equivalent (after hydrolysis) of a sample which had been twice distilled at 110–120°/766 mm. in a microstill. The ester (0·3517 g.) required 2·80 ml. of N-NaOH for complete hydrolysis giving an apparent equivalent of 125·5. (Calc. for *iso*butyl acetate, 116.) The value obtained would correspond with *iso*butyl acetate of about 93% purity.

Treatment of fraction II

Derivatives

(*a*) 2:4-*Dinitrophenylhydrazone.* Fraction II (0·203 g.) was dissolved in ethanol (3 ml.) and treated with a solution (200 ml., 0·4%) of 2:4-dinitrophenylhydrazine in approx. 2 N-HCl (Brady & Elsmie, 1926). This reagent is hereinafter referred to as Brady's reagent. The orange-yellow turbid mixture slowly flocculated and was filtered after keeping overnight. The orange-coloured product (0·200 g.) was recrystallized from light petroleum (b.p. 40–60°) giving orange needles of m.p. 87°. (Found: C, 54·9; H, 6·0; N, 18·2. Calc. for $C_{14}H_{18}O_4N_4$. C, 54·9; H, 5·9; N, 18·3%.) This gave a pure red colour with ethanolic NaOH and was evidently the hydrazone of a carbonyl compound, $C_8H_{14}O$. With the 2:4-dinitrophenylhydrazone of naturally occurring methylheptenone (m.p. 87°) prepared similarly it gave no depression in m.p. on admixture. Allen (1930) recorded a m.p. of 81° for this derivative, but gave no analytical figures. Assuming complete precipitation of the derivative, the crude yield would correspond to about 40% of methylheptenone in fraction II.

(*b*) *Semicarbazone.* Fraction II (0·230 g.) was heated on the water bath for 1 hr. with semicarbazide hydrochloride (0·50 g.) and Na acetate (1 g.), both dissolved in the minimum amount of water. Ethanol (2 ml.) was added to give a clear reaction mixture. On cooling colourless flattened needles were obtained, which after recrystallization from light petroleum (b.p. 80–100°), had m.p. 135–136°; yield 0·10 g. (Found: C, 59·2; H, 9·1; N, 21·8. Calc. for $C_9H_{17}ON_3$; C, 59·0; H, 9·4; N, 22·9%.) This would be derived from a carbonyl compound, $C_8H_{14}O$. The m.p. was not depressed on admixture with the authentic semicarbazone of methylheptenone, m.p. 135–136°. This m.p. agrees with that found by Hey & Morris (1948). A portion of fraction II therefore consists of 2-methylhept-2-en-6-one.

Fraction II therefore consisted to the extent of some 40% of methylheptenone, the remainder being presumably non-ketonic in nature and containing a substance or substances of similar b.p. and possessing an asymmetric carbon atom since the fraction was found to be optically active. It was considered that the most likely substance having these properties was the corresponding methylheptenol (or methylheptanol). A number of reagents likely to give crystalline products with the presumed hydroxyl group were tried: namely, 3:5-dinitrobenzoyl chloride, phenyl*iso*cyanate, α-naphthyl*iso*cyanate; conversion to the acid phthalate and preparation of crystalline esters was also attempted, but only non-crystalline oils were produced; success was finally attained by the use of cyanic acid to yield allophanates.

(*c*) *Allophanates.* Cyanic acid obtained by heating dry cyanuric acid (1 g.) distributed along a horizontal Pyrex tube (10 × 1 cm.) bent downward at the exit, was driven by a slow current of dry CO_2 into fraction II (0·50 g.) contained in a small bulb supported in a freezing mixture. When all the cyanuric acid had been decomposed, the bulb was removed from the freezing mixture and gently agitated. Reaction took place with considerable rise in temperature and the mixture solidified. After some hours the reaction mixture was dissolved in warm ether and any cyanuric acid present removed by filtration. The residue on removal of the ether consisted of a white slightly sticky solid, smelling of the unattacked methylheptenone. Trituration of the product with the minimum volume of cold light petroleum (b.p. 40–60°) removed both the stickiness and the odour and gave a white powder, m.p. 80–95° (0·35 g.), which was recrystallized from 40% (v/v) aqueous ethanol by warming and cooling to room temperature and gave mixed needle crystals (0·19 g.) of m.p. 115–123°.

The mother liquor, after chilling, deposited a homogeneous crop of crystalline tablets (38 mg.) of m.p. 102–103°, which formed microcrystalline sheaves of needles, m.p. 103–104°, on recrystallization from light petroleum (b.p. 40–60°). This substance was optically inactive. Admixture with an authentic sample of DL-methylheptenyl allophanate

of m.p. 103–104° gave no depression. Doeuvre (1929) records a m.p. of 99–100° for the racemic allophanate.

The main fraction (0·19 g.) on further purification showed a gradual rise in m.p. to a constant value. A final recrystallization from hot water gave material (35 mg.), of m.p. 136–137°, $[\alpha]_{5461}^{21°}$ +47° in ethanol. (Found: C, 55·9; H, 8·4; N, 13·1. $C_{16}H_{18}O_3N_2$ requires C, 56·0; H, 8·5; N, 13·1%.) The m.p. was not depressed on admixture with the allophanate of synthetic L-methylheptenol, m.p. 137°. $[\alpha]_{5461}^{20°}$ +47° in ethanol (*c*, 1·06). The appearance and behaviour of the two allophanates on recrystallization was of diagnostic value. The racemic allophanate, m.p. 103–104°, formed microcrystalline colourless needles which retained their shape on standing in water. In contrast, the dextrorotatory allophanate, m.p. 137°, formed microcrystalline feathery needles which appeared to have a slight blue tinge when viewed by reflected light. On standing in water they lost their crystalline form overnight and became completely gelatinous. When the substance was redissolved by heating, the needles were produced on cooling, eventually becoming gelatinous. The racemic form was slightly more soluble in both water and light petroleum.

No other allophanates were detected in the mother liquor.

Early clues as to the nature of the distillate fraction II which led to the identification of the substances present by means of suitable derivatives were afforded by ozonolysis and by the action of permanganate.

Ozonolysis. Fraction II (0·2370 g.) dissolved in $CHCl_3$ (20 ml.) and chilled in ice was treated with a current of ozonized O_2 for 40 min. The resulting ozonide was decomposed by shaking the $CHCl_3$ solution with an equal volume of water at 55° and separating the two layers.

The water layer was treated with Brady's reagent. The yellow precipitate (40 mg.; m.p. 190–205°) recrystallized from pyridine gave microcrystalline needles of m.p. 227°.

The $CHCl_3$ layer, after removal of solvent *in vacuo*, was treated with ethanol (3 ml.) and Brady's reagent (200 ml.). The product (0·32 g.) when recrystallized from pyridine, nitrobenzene, or xylene had m.p. 228–229° (0·10 g.) identical with the dinitrophenylhydrazone obtained from the water layer. (Found: C, 44·4; H, 3·5; N, 24·1. Calc. for $C_{17}H_{16}O_8N_8$: C, 44·4; H, 3·5; N, 24·4%.) The analysis indicates that the product is a *bis*-dinitrophenylhydrazone of $C_5H_8O_2$, which would be expected to give a blue colour with ethanolic KOH. This product however, when dissolved in methanol, gave a ruby-red colour on addition of a drop of 2N-KOH. The *bis*-dinitrophenylhydrazone of levulaldehyde of the same m.p. as the product, prepared for comparison, gave an identical colour reaction and showed no depression in m.p. on mixing the two substances.

The aqueous filtrate obtained from the above derivative slowly deposited an orange-brown *bis*-dinitrophenylhydrazone, giving a blue colour with methanolic KOH. Recrystallization from nitrobenzene gave orange-brown needles of m.p. 302–304° (decomp.) undepressed by the corresponding derivative of methylglyoxal. (Found: C, 42·1; H, 2·7; N, 27·5. Calc. for $C_{15}H_{12}O_8N_8$: C, 41·7; H, 2·8; N, 25·9%.)

In the later stages of distillation of the $CHCl_3$ from the decomposed ozonides a precipitate appeared in a trap containing Brady's reagent inserted in the vacuum line. This afforded a product which on recrystallization from ethanol formed yellow needles of m.p. 127–128°, undepressed on admixture with the dinitrophenylhydrazone of acetone, m.p. 128°. Ozonolysis thus gave rise to the products levulaldehyde, methylglyoxal, and acetone.

Oxidation with $KMnO_4$. Fraction II (0·430 g.), suspended in water (200 ml.), was oxidized by $KMnO_4$ (1·8 g. in 100 ml. water) at room temperature. The colour of the $KMnO_4$ eventually disappeared; the pH of the reaction mixture was 8·4.

Distillation of the total mixture at 15 mm. into Brady's reagent gave a yellow precipitate which recrystallization showed to be the dinitrophenylhydrazone of acetone, m.p. 128°. (Found: C, 45·6; H, 4·2; N, 22·8. Calc. for $C_9H_{10}O_4N_4$: C, 45·4; H, 4·2; N, 23·5%.) The reaction mixture was then filtered and acidified with H_2SO_4 and the volatile acid was distilled off *in vacuo*, and converted to the *p*-bromophenacyl ester which had m.p. 84–85° not depressed by authentic *p*-bromophenacyl acetate.

The colourless aqueous concentrate remaining after removal of the acetic acid was exhaustively extracted with ether. The extract was an oil containing a few crystals of oxalic acid. The oxalic acid was removed by dissolution of the oil in water and precipitation as calcium oxalate; the oil was recovered by ether extraction. The non-volatile acid present was converted to the *p*-bromophenacyl ester. The m.p. of the crude ester (35 mg.) was 130–140°, it was raised to 158–159° by recrystallization from light petroleum. (Found: C, 48·0; H, 4·0; Br, 26·2. $C_{12}H_{13}O_4Br$ requires C, 47·8; H, 4·4; Br, 26·6%.) The m.p. was not depressed on admixture with authentic *p*-bromophenacyl α-hydroxy-*iso*butyrate, m.p. 158–159°.

Oxidation with $KMnO_4$–CrO_3. Fraction II (0·507 g.) suspended in water (30 ml.) was oxidized by $KMnO_4$ (1 g., 10 ml. water), CrO_3 (0·6 g.) and aqueous H_2SO_4 (1 ml. conc. acid in 10 ml. of solution), by heating on a water bath (1 hr.). Volatile oxidation products were removed by vacuum evaporation. The chromium, after reduction to the green chromous salt by $NaHSO_3$, was removed by precipitation with excess aqueous Na_2CO_3, finally at 100°. The acidified aqueous residue was subjected to continuous ether extraction. The extract gave a 2:4-dinitrophenylhydrazone (0·23 g.) which, on recrystallization, formed orange needles of m.p. 203–204°, soluble in aqueous $NaHCO_3$ and giving no depression in m.p. on admixture with the 2:4-dinitrophenylhydrazone of levulic acid, m.p. 203–204°.

The products of ozonolysis of fraction II, namely methylglyoxal, levulaldehyde and acetone are recorded by Pummerer, Matthaus & Socias-Viñals (1936) as ozonization products of methylheptenone under similar conditions. These workers detected methylglyoxal only when excess of ozone was used.

Oxidation of fraction II with cold aqueous $KMnO_4$ gave rise to acetone, acetic acid, oxalic acid and α-hydroxy*iso*butyric acid. The last-named acid, which does not appear to have been previously recorded as an oxidation product of methylheptenone, no doubt arises by further oxidation of the 2-methylheptane-2:3-diol-6-one, which was found by Harries (1902) to be the primary oxidation product of methylheptenone by 2% $KMnO_4$.

The hot oxidation of fraction II with $KMnO_4$–CrO_3 according to the method of Tiemann & Semmler (1895) produced levulic acid, as recorded by these authors for methylheptenone.

Preparation of methylheptenol

Racemic 2-methylhept-2-en-6-ol was prepared by reduction of methylheptenone by Na in ethanol according to Rupe & Schlocoff (1905). The allophanate obtained from it had m.p. 103–104°. Doeuvre (1929) records the m.p. as 99–100°. (Found: C, 55·9; H, 8·5; N, 13·0. Calc. for $C_{16}H_{18}O_3N_2$: C, 56·0; H, 8·5; N, 13·1%.)

Resolution of racemic methylheptenol. This was achieved by crystallization of the brucine salt of the acid phthalate prepared from the methylheptenol, following the method of Levene & Haller (1929). The acid phthalate (67·3 g.) gave 35 g. of recrystallized brucine salt $[\alpha]_{5461}^{20°} - 4{\cdot}26°$ in ethanol (*c*, 7·8). This salt was reconverted to the acid phthalate, which was steam distilled with NaOH, giving 5·5 g. of (+)-methylheptenol, $[\alpha]_{5461}^{20°} + 19{\cdot}1°$. This, from its degradation by Levene & Haller (1929) to the dextrorotatory L-lactic acid, is the L-methylheptenol.

Allophanate. The allophanate of the L-methylheptenol had m.p. 137°, $[\alpha]_{5790}^{21°} + 41°$, $[\alpha]_{5461}^{21°} + 47°$ in ethanol (*c*, 1·06). (Found: C, 56·2; H, 8·3; N, 13·3; $C_{10}H_{18}O_3N_2$ requires C, 56·0; H, 8·5; N, 13·1%.)

The possibility was considered that methylheptenone and DL-methylheptenol might be artifacts formed by oxidation and racemization respectively of L-methylheptenol during the distillation process and not true metabolic products.

A sample of synthetic L-methylheptenol was heated at 200° for 30 min. and then distilled. These conditions are more drastic than those adopted in the fractionation of the metabolic products. During the heating a slight yellow colour developed, but the rotation of the colourless distillate (+7·89°) barely differed from that of the original material (+7·92°) measured under the same conditions. The difference is scarcely outside the limits of experimental error. Furthermore, the distillate gave no reaction with Brady's reagent, indicating the absence of appreciable amounts of methylheptenone. Thus all the products identified are genuine metabolic products of the fungus.

Products from *Endoconidiophora virescens*

The development of mycelium was somewhat slower than in the previous species and completely covered the medium after 6–7 days. The upper surface was dark grey (not mottled) with occasional dark-green areas. The reverse was black. Towards the end of the incubation period the odour was marked, but not of the same intensity nor as ester-like as that given by *E. coerulescens*.

Steam-volatile metabolic products. The distillation of the ether extract from the steam distillate gave only one fraction (fraction II) of approximately constant boiling point, corresponding to the higher boiling fraction from *E. coerulescens*. Fraction I, 0·94 g., b.p. 50–165°/767 mm. Fraction II, 3·00 g., b.p. 172–173°/767 mm.

During the collection of fraction I the temperature rose continuously. No indication was obtained of any *iso*butyl acetate produced by *E. virescens*. Both fractions gave precipitates with Brady's reagent; fraction II gave the same 2:4-dinitrophenylhydrazone and allophanates as those obtained from *E. coerulescens*, but the amounts showed considerable differences. An estimate of the amount of methylheptenone in the distillate was obtained from the amount of 2:4-dinitrophenylhydrazone formed (on the assumption that precipitation is complete). The amount of L-methylheptenol was estimated from the rotation; the rest of the material was assumed to be DL-methylheptenol. These figures shown in Table 1 give a rough guide to the quantitative relationships of the higher boiling constituents of the two fungi.

Table 1. *Relative proportions of the metabolic products in the boiling range of* 172–176° *from* Endoconidiophora coerulescens *and* E. virescens

	Methylheptenone (%)	L-Methylheptenol (%)	DL-Methylheptenol (by difference) (%)
E. coerulescens	41	17	42
E. virescens	58	5	37

Products from *Endoconidiophora fimbriata*

Growth was much slower than in both the preceding species. The mycelium was greyish white with occasional black areas and a light grey reverse. A very faint odour could be detected through the cotton-wool plug, but this bore no resemblance to that previously encountered. Only a trace of steam-volatile products was obtained and this gave no characteristic dinitrophenylhydrazone or reaction for carboxylic esters.

SUMMARY

1. *Endoconidiophora coerulescens* Münch when grown on a liquid malt medium afforded the volatile metabolic products *iso*butyl acetate, 2-methylhept-2-en-6-one, and a mixture of L- and DL-2-methylhept-2-en-6-ol.

2. Under similar conditions, *E. virescens* Davidson produced the same methylheptenone and mixture of L- and DL-methylheptenol as *E. coerulescens*, but in different proportions. No *iso*butyl acetate was detected in this case.

3. No identifiable volatile metabolic products were obtained from *E. fimbriata* (Ell. & Hals.) Davidson grown under the same conditions.

4. The products identified are responsible for the odours of the fungi in culture.

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The Metabolism of the Oral Flora

I. THE OXYGEN UPTAKE AND ACID PRODUCTION BY MIXED HUMAN SALIVA IN THE PRESENCE AND ABSENCE OF GLUCOSE

By R. L. HARTLES and NORMA D. McDONALD
Biochemistry Department, School of Dental Surgery, University of Liverpool

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The early work of Miller (1890) has remained the foundation upon which many theories concerning the aetiology of dental caries have been based. Miller considered that caries was due to the dissolution of the inorganic portion of the enamel and dentine by acids. These acids, he postulated, were produced by the bacterial fermentation of food particles trapped in pits and fissures of the teeth which were not self cleansing. The main requirement of this theory is therefore that acid shall be produced in sufficient quantity, locally, to lower the pH to the point where dissolution of the enamel may occur.

The most common mouth organism which is capable of producing acid (acidogenic) and existing at a comparatively low pH (aciduric) is *Lactobacillus acidophilus*. This organism is accepted by many workers as a factor in the production of caries (Bunting, 1934; Snyder & Teachout, 1942). It also appears that a diet high in fermentable carbohydrate favours the development of this organism in the mouth (Arnold & McClure, 1941); such a diet also results in an increase in the incidence of dental caries (Jay, Hadley, Bunting & Koehne, 1936). Whether there is a causative relationship between these two observations remains to be proved. Other workers have cited streptococci and staphylococci (Anderson & Rettger, 1937; Florestano, 1942; Bibby, Volker & Van Kesteren, 1942) as being capable of producing acid. Fosdick, Hansen & Wessenger (1937) have shown that certain yeasts are also acidogenic, and that yeast and lactobacillus together produce acid more rapidly than either organism alone. It would seem dangerous therefore to consider one organism to be of paramount importance in a complex and varied flora such as exists in saliva; the various micro-organisms which are present may act in concert in a manner differing from their behaviour individually. It is important, as Fosdick, Campaigne & Fancher (1941) have pointed out, to consider not only the bacteria themselves but the enzymes which they contain.

More recently, doubt has been cast upon the validity of the 'acidogenic' concept of caries initiation, and a different theory has been advanced, namely that the primary lesion is due to the action of proteolytic enzymes upon the organic matrix of the enamel (Nuckolls & Frisbie, 1946; Frisbie & Nuckolls, 1947; Gottlieb, 1947).

Since the metabolism of the whole oral flora may be involved in the carious process it is possible that the study of the saliva as a 'biological unit' may prove to be of value.

It was decided therefore to examine the behaviour of various salivas making use of the Warburg manometric technique. It is realized, of course, that the salivary flora is only a sample, and possibly only a component, of the total oral flora.

In the present work determinations have been made of the oxygen consumption and lactic acid production by the saliva when incubated at 38° in the presence and absence of glucose. In addition, the lactic acid and total acid produced under anaerobic conditions have been determined with and without the addition of glucose.

The object of this preliminary investigation was to observe the magnitude and variation of oxygen consumption and lactic acid production in the salivas of subjects with varying degrees of dental caries, and to see whether these factors bore any relation to the caries experience of the subjects. The individuals included one completely caries free, one virtually free (one minor filled cavity), two with slight caries activity and five with a considerable number of carious lesions.

EXPERIMENTAL

Material. Samples of saliva were obtained from nine student volunteers, five men and four women, with varying caries experience, one of the men being completely free from dental caries.

Subject	Age (yr.)	Teeth decayed, missing or filled (D.M.F.)
J.C. ♂	26	19
D.R. ♂	22	16
M.S. ♀	27	19
A.M. ♀	22	23
H.F. ♂	26	17
R.B. ♂	19	4
S.H. ♀	19	4
Y.C. ♀	21	1
M.T. ♂	36	0

Collection of saliva. Samples (5 ml.) were collected by the subjects themselves each morning immediately on awakening whilst still lying in bed. The flow of saliva was stimulated in all cases by the chewing of paraffin wax (British Drug Houses Ltd., m.p. 48–49°). The subjects were instructed to make the wax scour all surfaces of the teeth. The saliva was collected in a sterile screw-capped bottle in as short a time as possible. The samples were brought to the laboratory within 2 hr. of collection.

Manometry. The samples of saliva (0·5 ml.) were buffered with 0·2 M-phosphate buffer, pH 7·4. Oxygen consumption was measured in Warburg manometers at 38°.

The final concentration of glucose, when used, was 0·005 M.

Lactic acid production. The lactic acid in the manometer contents was determined by the method of Barker & Summerson (1941) as described by Umbreit, Burris & Stauffer (1945).

Total acid production. The total acid was measured manometrically by the evolution of CO_2 from a bicarbonate buffer in an atmosphere of N_2 containing 5% CO_2.

Bacterial multiplication. Since times of up to 2 hr. elapsed between collection of salivary samples and their arrival at the laboratory, followed by a period of 1 hr. at 38°, it was important to know the extent of bacterial multiplication in these circumstances. It was not possible to carry out tests simultaneously on the nine subjects. Saliva from six further subjects was therefore collected and allowed to incubate with and without glucose at room temperature (15°) and at 38°. Counts were done at 30 min. intervals for 4 hr.

Non-cellular oxidation. It was also important to know whether the oxidative processes in saliva were due entirely to bacteria and other cells, or whether salivary enzymes were involved. For this purpose samples of saliva were filtered through a Seitz filter and the filtrate used in the manometer.

RESULTS

The mean figures quoted in the Tables represent the average of from ten to twenty independent observations made on different days.

The uptake of oxygen is expressed as μl. of O_2 taken up by 1 ml. saliva in 1 hr. There is a wide day-to-day variation in all subjects both with and without the addition of glucose. The results are given in Table 1.

Lactic acid and total acid production are expressed as micro-equivalents of acid produced by 1 ml. saliva in 1 hr. The results for aerobic conditions are presented in Table 2, those for anaerobic conditions in Table 3.

Bacterial multiplication. Saliva from six further subjects was obtained and counts made at half-hourly intervals during incubation with and without glucose at 15 and 38°. After 2 hr. at 15° with or without glucose there was only a slight increase in count, even after incubation with glucose for 2 hr. at 38°, the count was less than double the original figure.

Non-cellular oxidation. Saliva which had been filtered through a Seitz filter did not take up oxygen either in the absence or presence of glucose.

DISCUSSION

The comparison of respiratory rates and acid production on a volumetric basis, i.e. uptake of O_2 or acid produced/ml. saliva/hr., seems a reasonable course to adopt since the same volume of saliva was collected each morning under the same conditions. In future investigations it may be desirable to adopt another standard for comparison, e.g. O_2 consumption/mg. N, but for the present the volumetric basis will serve.

The bacterial multiplication under the conditions of the experiment appears to be of a low order and will not unduly influence the results. Since there is no evidence of any oxidation due to non-cellular constituents of the saliva, it would seem that the total oxidation is due to the contained cells.

Aerobic oxidation. The results indicate that there is a great variation in the mean O_2 consumption of the nine subjects. The oxygen taken up in the absence of added glucose was considerable, being greatest in the case of J. C. (caries active) and Y. C. (caries free). Lactic acid was not produced in detectable amounts by any of the salivas, which suggests that if carbohydrate were a substrate, it was

Table 1. *Oxygen consumption of saliva samples*

(All values are μl. O_2/ml. saliva/hr. Temp. 38°.)

Subject	Teeth decayed missing or filled	Without added glucose		With added glucose (0·005 M)		Difference between means with and without glucose ($b - a$)
		Mean (a)	Range	Mean (b)	Range	
J.C.	19	309	134–465	605	256–927	296
D.R.	16	184	94–336	350	144–685	166
M.S.	19	137	70–320	267	161–524	130
A.M.	23	96	40–135	157	97–292	61
H.F.	17	132	72–192	220	80–409	92
M.T.	0	56	32–76	187	112–254	137
Y.C.	1	315	230–384	385	304–490	70
S.H.	4	124	96–160	223	176–272	99
R.B.	4	157	120–188	237	205–257	80

Table 2. *Lactic acid production by saliva in aerobic conditions*

(Results are expressed in μg. or μequiv. lactic acid/ml. saliva/hr. Temp. 38°.)

Subject	Teeth decayed, missing or filled	Without added glucose	With added glucose (0·005M)		
			μg. lactic acid		μequiv. lactic acid
			Mean	Range	Mean
J.C.	19	Not detectable	100	75–160	1·1
M.S.	19	,,	287	210–385	3·2
A.M.	23	,,	191	138–270	2·1
H.F.	17	,,	183	75–480	2·0
M.T.	0	,,	819	240–1440	9·1
Y.C.	1	,,	165	130–190	1·8
S.H.	4	,,	140	100–175	1·6
R.B.	4	,,	125	100–165	1·4

Table 3. *Acid production by saliva under anaerobic conditions*

(Results expressed as μl. CO_2 or μequiv. acid/ml. saliva/hr. Temp. 38°.)

Subject	Teeth decayed, missing or filled	Total acid						Lactic acid	
		Without added glucose			With added glucose (0·005M)			Without glucose μequiv.	With glucose μequiv.
		μl. CO_2 produced		μequiv.	μl. CO_2 produced		μequiv.		
		Mean	Range	Mean	Mean	Range	Mean	Mean	Mean
J.C.	19	161	102–260	7·2	756	653–859	33·8	Nil	4·3
M.S.	19	140	100–200	6·3	645	490–803	28·8	,,	3·9
A.M.	23	215	136–356	9·6	450	348–560	20·5	,,	5·9
H.F.	17	140	60–224	6·3	646	539–689	28·8	,,	3·5
M.T.	0	174	120–328	7·8	704	568–765	31·4	,,	3·6
Y.C.	1	196	160–260	8·8	697	550–844	31·1	,,	6·2
S.H.	4	148	120–220	6·6	500	426–578	22·3	,,	7·1
R.B.	4	260	160–320	11·6	552	509–636	24·6	,,	8·6

being completely oxidized. The possibility must be considered that this O_2 consumption in the absence of added glucose may be due to the oxidation of products of proteolysis. In the subjects with active caries, substrate may be provided from the cavity contents, but in the caries-free cases the probability is that oxidation of a normal salivary constituent is taking place. This problem warrants further investigation. The addition of glucose caused an increased O_2 uptake in all cases and lactic acid was present in the manometer contents. There is a slight indication that the absolute increase in O_2 consumption due to the addition of glucose is greatest in the saliva of those subjects with most caries but, except in the case of J.C., there is no marked difference.

Since the addition of glucose caused the appearance of lactic acid (a typical product of anaerobiosis) as well as an increased O_2 uptake, it would appear that both anaerobic and aerobic processes are occurring side by side in the saliva. A greater O_2 uptake does not seem to reduce the quantity of lactic acid produced. This may be due to a differing cell content, but a factor which cannot be excluded is that vigorous aerobiosis may result in local O_2 lack and the encouragement of pockets of anaerobic activity. This is a factor which would be more apparent in the mouth than in an *in vitro* experiment.

An interesting observation was that the saliva of subject M.T., who was absolutely caries free, produced a vastly greater amount of lactic acid than any of the others (Table 2). In another subject (Y.C.), who was virtually caries free, the lactic acid produced was no less than in other subjects with a high incidence of caries (J.C., H.F. and A.M.). It would appear therefore that a high lactic acid production is not necessarily associated with a high degree of caries, and that conversely caries may develop when the acid production is low.

Anaerobic oxidation. Since it is possible that anaerobic conditions exist locally in the mouth, arising perhaps where aerobes are using up all the available O_2 in a dental plaque, the study of anaerobic oxidation in the saliva is pertinent. When the salivas were incubated without added glucose in a bicarbonate buffer at 38° no detectable lactic acid was formed in any of the subjects, but acid of some kind was nevertheless produced in varying amounts. In this case the acid produced by the caries-free subjects was no less than that produced by the others (Table 3). The origin and nature of this acidic material is obscure and requires further study. The addition of glucose caused in all subjects a large increase in the total acid production, the caries-free subjects forming just as much as the caries-active.

The lactic acid formed in the presence of glucose is greater under anaerobic than aerobic conditions except in the case of subject M.T. The fact which does emerge, however, is that the potential acid production in the most favourable circumstances, i.e. lack of O_2, is no less in the caries-free subjects than in those with active caries. A further point worthy of emphasis is that the lactic acid produced by incubation with glucose under anaerobic conditions accounts for only one-sixth to one-third of the total acid formation.

A great mass of evidence has been put forward to support the contention that acid production in the mouth is associated with the incidence of dental caries, and various caries-susceptibility tests have been elaborated on this basis, a notable one being due to Fosdick, Hanson & Epple (1937).

Stephan (1944) studied the changes occurring in the intra-oral pH, after a rinse with a 10% aqueous glucose solution; his general conclusion was that the greatest fall in pH occurred in subjects with most extensive caries. He did report, however, that bacterial plaques, which are capable of producing acid rapidly, grow not only on the teeth of individuals who have active caries, but also on the teeth of those who are caries free.

Volker & Pinkerton (1947) have shown that various unrefined carbohydrates, including starch and dextrin, are converted to acid by saliva at rates similar to those observed for refined sugars. If acid production is of major importance in the initiation of caries, it is difficult to reconcile this observation with the widely held view of the specific cariogenic action of refined sugars. Osborn, Noriskin & Staz (1937) reported that tooth surfaces exposed to mixtures of saliva and unrefined carbohydrates were decalcified to a less degree than those immersed in saliva and refined sugars. They suggested that this was due to the removal during the refining process of constituents which protect the enamel against dissolution, but were unable to say more about the nature of this protection.

We are aware that the mere production of a given quantity of acid may result in a different effect in different subjects. Other factors will exert an influence upon the change in pH, notably buffering capacity and rate of flow of the saliva.

Claims have been made to correlate the buffering power of the saliva and caries experience (Dreizen, Mann, Cline & Spies, 1946). Against this, there is no evidence to show a relationship between salivary phosphate (an important buffer) and caries (Deakins, Cheyne, Bibby & Van Kesteren, 1941). Stralfors (1948) has shown that the buffering capacity of the bacterial plaques occurring on the tooth surface is greater than that of the saliva from the same mouth.

The case of subject M.T. was the more remarkable in that the teeth of both jaws were somewhat crowded together, there being in fact a supernumerary in the upper jaw. Such orthodontic conditions are usually considered to predispose to the development of dental caries, especially of the interstitial kind (Thoma, 1944).

The relative importance of all the various factors involved is obscure in the present state of knowledge, but there is some evidence to suggest that acid production *per se* is not of paramount importance in the aetiology of caries. The results of this investigation, limited though it is by the difficulty of obtaining caries-free subjects, tends to support this point of view. It appears then, that whilst acid production in the mouth may be a necessary concomitant in the carious process it is not in itself a sufficient cause.

SUMMARY

1. The oxygen uptake of saliva samples, with and without the addition of glucose, has been studied in nine subjects, of whom one was absolutely free from dental caries and another virtually so.

2. The absolute oxygen consumption varied from subject to subject, and also from day to day in the same individual. There was no obvious relation between oxygen uptake and dental caries experience.

3. The amount of lactic acid produced by the salivas in this investigation bears no relation to the degree of caries in the subjects.

4. The greatest production of lactic acid in aerobic circumstances was by a subject who was absolutely caries free.

5. The total acid and lactic acid produced in the absence of oxygen bear no apparent relation to the caries incidence.

6. It is suggested that acid production *per se* is not a sufficient cause for the production of dental caries.

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Metabolism of Polycyclic Compounds

6. CONVERSION OF PHENANTHRENE INTO DIHYDROXYDIHYDROPHENANTHRENES

BY E. BOYLAND AND G. WOLF

The Chester Beatty Research Institute, The Royal Cancer Hospital, London, S.W. 3

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Bergel & Pschorr (1903) showed that rabbits dosed with phenanthrene excrete a phenolic derivative, phenanthrylglucuronide. Investigation of the metabolism of anthracene in rats and rabbits (Boyland & Levi, 1935) and of naphthalene (Young, 1947; Booth & Boyland, 1947, 1949) has shown that these hydrocarbons are transformed into dihydroxydihydro derivatives. Such diols are readily converted to phenols by dehydration in the presence of acid, and it is possible that some of the phenolic metabolic products which have been isolated are decomposition products of dihydroxydihydro derivatives. In the present paper this reaction of diol formation, which has been called perhydroxylation because it involves addition of the elements of hydrogen peroxide, is shown to take place with phenanthrene.

Young (1947) reported the isolation of a crystalline product from the urine of rats dosed with phenanthrene. This product with m.p. 186–187° and analysis corresponding to a formula $C_{14}H_{12}O_2$ would appear to be identical with the 9:10-dihydroxy-9:10-dihydrophenanthrene characterized below. Criegee, Marchand & Wannowius (1942) prepared *cis*-9:10-dihydroxy-9:10-dihydrophenanthrene by oxidation of phenanthrene with osmic acid. Attempts to synthesize *trans*-9:10-dihydroxy-9:10-dihydrophenanthrene by published methods (e.g. Skita, 1925) or by oxidation of phenanthrene have so far been unsuccessful, but reduction of phenanthraquinone with lithium aluminium hydride by the method described by Finholt, Bond & Schlesinger (1947) has given the *trans* compound identical with this metabolite (Booth, Boyland & Turner, 1950).

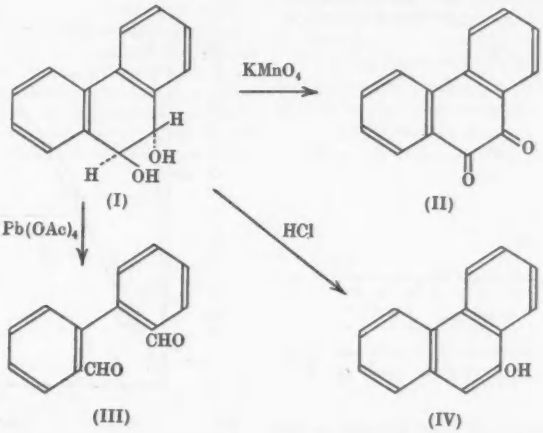
EXPERIMENTAL

A solution of phenanthrene (20%, w/v) in arachis oil was injected intraperitoneally into rats and rabbits. Rats were dosed with 10 ml./kg. body weight and rabbits with 5 ml./kg. body weight twice weekly and showed no ill effects from the treatment. The fresh urine which was collected in metabolism cages was extracted with ether as soon as possible. The ethereal extract was evaporated to dryness. In some experiments, the residue could be crystallized from benzene without further treatment. When the material was too impure for direct crystallization, it was dissolved in methanol containing 1% (v/v) water

and this solution extracted in a continuous extractor with light petroleum, b.p. 40–60°. Crystalline material separated from the light petroleum extract and was further purified by recrystallization from benzene after decolorization with charcoal.

Product from rat urine

9:10-*Dihydroxy*-9:10-*dihydrophenanthrene*. The compound from rat urine was dimorphic and crystallized from benzene as long, silky colourless needles, m.p. 185–187° (Found: C, 79·1; H, 5·6. $C_{14}H_{12}O_2$ requires C, 79·2; H, 5·6%), and as prisms, m. p. 194° (Found: C, 79·2; H, 5·7%). It was soluble in water and hexane and easily soluble in ethanol. This product is probably identical with that described by Young (1947). It had the same melting points as the two forms of the compound obtained synthetically by Booth *et al.* (1950). The products isolated from urine in this way were usually optically inactive, but material with *laevo* rotation was sometimes obtained. The problem of the optical activity is still under investigation.



Acetylation. Specimens (0·05 g.) of the diol were dissolved in pyridine (0·05 ml.) and acetic anhydride (0·4 ml.), and kept at room temperature for 16 hr. After neutralization of the pyridine with N-HCl the reaction products were extracted with ether and the extract shaken with M-Na_2CO_3 to remove excess acetic anhydride. The ether extracts were then shaken with water, dried with anhydrous Na_2SO_4 and evaporated to dryness. The *diacetate* crystallized from light petroleum (b.p. 40–60°) in colourless elongated prisms and was recrystallized from ethanol, m.p. 173°, alone or mixed with a synthetic specimen (Found: C, 73·0; H, 5·4. $C_{18}H_{16}O_4$ requires C, 72·9, H, 5·4%). The compound was sparingly soluble in water or cold ethanol but soluble in ether and benzene.

Dehydration of 9:10-*dihydroxy*-9:10-*dihydrophenanthrene*. When the diol was heated in 2 N-HCl at 100° for 2 hr., 9-phenanthrol (IV), m.p. 153° (alone and when mixed with an authentic specimen) was formed. This compound, obtained from the diol, was dissolved in NaOH and shaken with dimethyl sulphate, when it gave 9-methoxyphenanthrene, m.p. 96° (alone and when mixed with an authentic specimen). When treated with pyridine and acetic anhydride, the phenanthrol derived from the optically active diol yielded 9-acetoxyphenanthrene, m.p. 77°, alone and when mixed with an authentic specimen (Found: C, 80·8; H, 5·1. Calc. for $C_{16}H_{12}O_2$: C, 81·2; H, 5·0%).

Oxidation of 9:10-*dihydroxy*-9:10-*dihydrophenanthrene*. (*a*) By $KMnO_4$: 15 ml. of 0·25 M-$KMnO_4$ was added to the diol (0·05 g.) in water (10 ml.) and the mixture heated at 100° for 1 hr. The solution was acidified with 10 N-HCl and extracted with a large volume of ether. The extract was washed with N-NaOH and water. After drying and evaporation of the solvent, phenanthraquinone (II) separated and was recrystallized from ethanol, m.p. 208°, alone and when mixed with an authentic specimen (Found: C, 80·2; H, 4·0. Calc. for $C_{14}H_8O_2$: C, 80·6; H, 3·9%).

(*b*) By Pb tetraacetate: the diol (0·025 g.) was added to a solution of Pb tetraacetate (0·1 g.) in anhydrous benzene (10 ml.) and kept at room temperature for 8 hr. The solution was then washed, first with N-HCl to remove the excess Pb tetraacetate, then with M-Na_2CO_3, and filtered to remove the insoluble Pb salt. After evaporation of the solvent, diphenyl-2:2′-dialdehyde (III) separated and was crystallized from light petroleum (b.p. 40–60°), m.p. 64° (alone and when mixed with an authentic specimen, prepared by oxidation of synthetic *cis*-9:10-diol with Pb tetraacetate according to Criegee *et al.* 1942). The dioxime was prepared and crystallized from aqueous methanol as colourless needles, m.p. 183–184°.

Spatial configuration. The hydroxyl groups appear to have the *trans* configuration as indicated by the following pieces of evidence.

(*a*) Identity with the synthetic *trans* compound.

(*b*) Difference from synthetic *cis* derivative in physical properties (Table 1).

(*c*) Differences in rate of oxidation of *cis* and *trans* diols. Lead tetraacetate oxidation was carried out in glacial acetic acid as described for naphthalene derivatives (Booth & Boyland, 1949).

Table 1. *Properties of* 9:10-*dihydroxy*-9:10-*dihydrophenanthrenes*

	Cis form (synthetic)	*Trans* form (synthetic)	Natural product
M.p. of diol	178°	185–187° and 194°	185° and 194°*
M.p. of diacetate	110°	173°	173°†
Oxidation with lead tetraacetate:			
Time for half reaction at 20° (min.)	4·2	0·3	0·3
$k_{20°}$ (t = min.)	13·8	130	130
Dehydration with N-HCl:			
Time for half reaction at 100° (min.)	0·85	11·5	12·0
$k_{100°}$ (t = min.)	8·1	0·60	0·58

* Mixed m.p. of natural product and synthetic *trans* form of m.p. 185–187° was 184–185°.
† Mixed m.p. of natural product and synthetic *trans* compound 173°.

The rates of reaction of the natural 9:10-dihydroxy-9:10-dihydrophenanthrene and synthetic *cis* and *trans* forms of 9:10-dihydroxy-9:10-dihydrophenanthrene with Pb tetraacetate are shown in Fig. 1 and Table 1. Under these conditions phenanthrene itself was not oxidized. The natural product was oxidized much more rapidly than the synthetic *cis* compound.

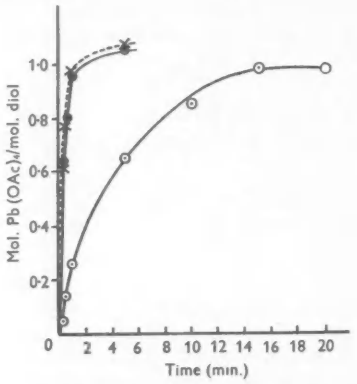


Fig. 1. Oxidation of phenanthrene diols with lead tetraacetate. Natural, ×--×; synthetic *trans*, ●—●—; *cis*, ⊙—⊙—.

(*d*) Rates of dehydration with acid. Solutions of the natural 9:10-dihydroxy-9:10-dihydrophenanthrene and the synthetic *cis* and *trans* compounds were heated in N-HCl at 100°. The amount of 9-phenanthrol formed was estimated colorimetrically by coupling with diazotized sulphanilic acid in alkaline solution. The rates of phenol formation of the two isomers are shown in Fig. 2 and Table 1.

(*e*) Colour reaction with potassium triacetyl osmate. With this reagent (Criegee *et al.* 1942) synthetic *cis*-9:10-dihydroxy-9:10-dihydrophenanthrene gave a positive reaction, while 9:10-dihydroxy-9:10-dihydrophenanthrene from rat urine, or the synthetic *trans* compound, did not react.

(*f*) Ultraviolet absorption spectrum. Ultraviolet absorption measurements made by Mr R. N. Beale and Dr E. M. F. Roe and to be published separately, show the natural 9:10-dihydroxy-9:10-dihydrophenanthrene to have a similar absorption to that of synthetic *trans*-9:10-dihydrophenanthrene.

Product from rabbit urine

The crystals obtained from the extract had an indefinite melting point and were shown to be a mixture of diols. The mixture was acetylated with cold acetic anhydride and pyridine. The mixed

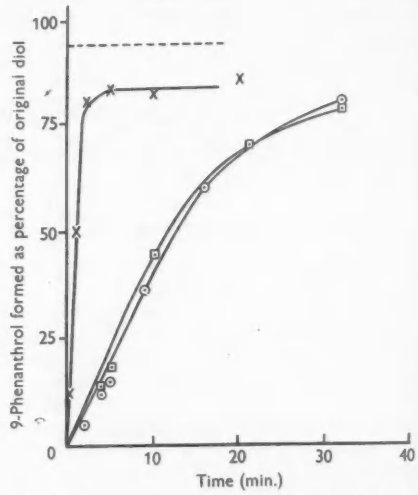


Fig. 2. Elimination of water from isomeric 9:10-dihydroxy-9:10-dihydrophenanthrenes in N-HCl at 100°. Theoretical yield, - - - - -; synthetic *cis* compound, ×—×—; natural product, ⊙—⊙—; synthetic *trans* compound, ⊡—⊡—.

acetates were dissolved in light petroleum (b.p. 40–60°) with a little ether, and fractionally crystallized. The first crop consisted of the diacetate of *trans*-9:10-dihydroxy-9:10-dihydrophenanthrene, m.p. 173° (alone and when mixed with a specimen of the compound obtained from rat urine). By treatment with N-KOH in methanol at the boiling point for 2 min., 9:10-dihydroxy-9:10-dihydrophenanthrene (I) was obtained, m.p. 185–187° (alone and when mixed with a specimen of the compound ob-

tained from rat urine). This was identical with the compound obtained from rat urine in every respect.

The second crop of crystals consisted of the *diacetate* of 1:2-*dihydroxy*-1:2-*dihydrophenanthrene* crystallizing from ethanol in colourless, short, thick needles, m.p. 106° (Found: C, 72·8; H, 5·4. $C_{18}H_{16}O_4$ requires C, 72·9; H, 5·4%). Optical rotation $[\alpha]_D^{20°} = -380°$ (*c*, 0·4% in ethanol).

laevo-1:2-*Dihydroxy*-1:2-*dihydrophenanthrene* (V). The above diacetate was dissolved in N-KOH in methanol and the solution boiled for 2 min. and poured into water. The diol was then extracted with ether and crystallized from benzene in colourless thick prisms, m.p. 155–159° (Found: C, 79·1; H, 5·6. $C_{14}H_{12}O_2$ requires C, 79·2; H, 5·6%). The compound is soluble in water, benzene and light petroleum, and easily soluble in ethanol. Optical rotation $[\alpha]_D^{20°} = -240°$ (*c*, 0·5% in ethanol).

Dehydration (*Method a*). *laevo*-1:2-Dihydroxy-1:2-dihydrophenanthrene (0·02 g.) was dissolved in 0·25 N-HCl in methanol and the solution boiled for 30 min. Water was then added, the resulting mixture of phenols extracted with ether, the ether extract washed with M-$NaHCO_3$ and dried over Na_2SO_4. After evaporation of the solvent, the product was dissolved in pyridine and acetic anhydride and heated at 100° for 3 hr. After removal of the pyridine with N-HCl, the mixture was dissolved in ether and, after washing with M-$NaHCO_3$ and drying over Na_2SO_4, evaporated to dryness. The resulting solid was dissolved in light petroleum (b.p. 60–80°), and by slow crystallization, a product m.p. 168–169° was obtained. This is now being further investigated. The residue from the mother liquors was dissolved in hot N-NaOH and treated with successive portions of dimethyl sulphate until the fluorescence of the solution had changed from green to blue. The solids obtained were extracted with ether, the extracts washed with water, dried over Na_2SO_4 and the solvent evaporated. The residual solid was dissolved in light petroleum (b.p. 40–60°) and chromatographed on a column of Al_2O_3 (Savory & Moore Ltd.) made with the same solvent. Two fluorescing bands were seen in ultraviolet light; the less strongly adsorbed band, on elution, was found to be 1-methoxyphenanthrene (VI), m.p. 105°, alone and when mixed with an authentic specimen (Found: C, 85·9; H, 5·8. Calc. for $C_{15}H_{12}O$: C, 86·4; H, 5·8%).

Dehydration (*Method b*). *laevo*-1:2-Dihydroxy-1:2-dihydrophenanthrene (0·02 g.), when suspended in 2 N-HCl and heated to 100° for 1 hr., gave 1-hydroxyphenanthrene, m.p. 156° (alone and when mixed with an authentic specimen). This on treatment with dimethyl sulphate in NaOH solution as described above, gave 1-methoxyphenanthrene (VI), m.p. 105° (alone and when mixed with an authentic specimen).

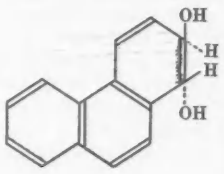
Hydrogenation. *laevo*-1:2-Dihydroxy-1:2-dihydrophenanthrene takes up Br_2 from a solution in $CHCl_3$ and can be hydrogenated. The diacetate of the diol (0·15 g.) in ethanol (10 ml.) was treated with Pt (Adams's catalyst) and hydrogenated at atmospheric pressure and room temperature. The volume of H_2 taken up was 7·3 ml. When absorption of H_2 ceased, the catalyst was removed by filtration and the solvent evaporated. The *diacetate* of 1:2-*dihydroxy*-1:2:3:4-*tetrahydrophenanthrene* (VII) crystallized in colourless needles from ethanol, m.p. 112° (Found: C, 72·0; H, 6·5. $C_{18}H_{18}O_4$ requires C, 72·4; H, 6·1%). The compound is optically active, $[\alpha]_D^{20°} = -105°$ (*c*, 0·6% in ethanol).

Steric configuration. The result of the potassium triacetyl osmate colour reaction of Criegee *et al.* (1942) on the 1:2-dihydroxy-1:2-dihydrophenanthrene was negative, which would indicate that the hydroxyl groups in the compound have the *trans* configuration.

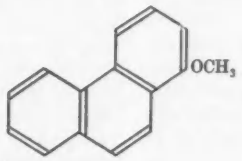
DISCUSSION

The experiments show that phenanthrene is metabolized to dihydroxydihydro derivatives by rats and rabbits. The presence of two hydroxyl groups is shown by the formation of diacetates. That the hydroxyl groups are on adjacent carbon atoms is shown by the ease of oxidation with lead tetraacetate to dialdehydes.

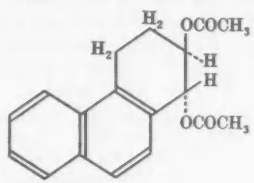
That the hydroxyl groups in the derivative isolated from rat urine must be in the 9:10 positions is shown by (*a*) the oxidation to 9:10:phenanthraquinone (II) with potassium permanganate, (*b*) the oxidation to diphenyl-2:2′-dialdehyde (III) with lead tetraacetate, and (*c*) the conversion to 9-phenanthrol on treatment with hot acid. That the hydroxyl groups have the *trans* configuration is indicated by the fact that the derivative differs from the synthetic *cis* compound in several respects, viz.: (1) melting point, (2) not giving an acetone derivative, (3) reacting with lead tetraacetate at a faster rate, (4) not giving the colour reaction with potassium triacetyl osmate, (5) being dehydrated more slowly to 9-phenanthrol. This evidence for the structure and the *trans* configuration was obtained before the synthetic compound was available. The identity with the synthetic compound proves the structure previously assigned to the compound.



(V)



(VI)



(VII)

The experiments are of interest in that in this compound *trans*-9:10-dihydroxy-9:10-dihydrophenanthrene is more rapidly oxidized by lead tetra-

acetate than is the *cis* derivative. This would appear to be an exception to Criegee's rule (Criegee, Kraft & Rank, 1933) that *cis* glycols are more rapidly oxidized than the corresponding *trans* compounds. The evidence seems quite clear in the case of these 9:10-diols derived from phenanthrene. Another similar anomaly is the oxidation of the *trans*-9:10-dimethyl-9:10-dihydroxyphenanthrene which Criegee (personal communication) has found to react with lead tetraacetate more rapidly than the corresponding *cis* derivative.

As the natural optically inactive 9:10-dihydroxy-9:10-dihydrophenanthrene has been shown to be the *trans* compound it must be a racemic form. The racemate is possibly formed by racemization of the optically active material after excretion.

One of the products isolated from rabbit urine is identical with the *trans*-9:10-dihydroxy-9:10-dihydrophenanthrene isolated from rat urine. The other diol from rabbit urine is shown to be *trans*-1:2-dihydroxy-1:2-dihydrophenanthrene (V). The evidence for this structure depends upon the following observations: (*a*) the conversion to 1-phenanthrol on treatment with hot, aqueous acid; (*b*) the formation of 1-phenanthrol on treatment with acid in methanol; (*c*) the formation of 1:2-dihydroxy-1:2:3:4-tetrahydrophenanthrene (VIII) by catalytic hydrogenation. The only evidence for the configuration of the hydroxyl groups is the fact that the compound does not discharge the blue colour of potassium triacetyl osmate. In the absence of contrary evidence this would indicate that the compound is *laevo-trans*-1:2-dihydroxy-1:2-dihydrophenanthrene.

The results are of interest in relation to those obtained with other hydrocarbons. Phenanthrene differs from naphthalene and anthracene in having one bond with high reactivity. In naphthalene and anthracene there are four equivalent bonds all of equal reactivity. If in the metabolism of anthracene or naphthalene the hydrocarbons are attached to an enzyme through a 3:4 bond by an addition reaction, the adjacent double bond (1:2) would then become more reactive so that oxidation might readily take place in this 1:2 position. Thus a 1:2-dihydroxy-1:2-dihydro-3:4-'tissue'-complex may be formed which breaks down to give the 1:2-dihydroxy-1:2-dihydro derivative of the hydrocarbon.

The formation of the 1:2-dihydroxy-1:2-dihydrophenanthrene is analogous to the metabolism of the more complex hydrocarbons such as 1:2-benzanthracene and 1:2:5:6-dibenzanthracene in that the addition occurs in positions of secondary chemical activity. The region corresponding to the phenanthrene 9:10 double bond, which is the region with the highest charge of electrons, has been called the '*K* region' by French workers (cf. Pullman & Pullman, 1946). If the bond of the *K* region is saturated, because the hydrocarbon is held to a tissue constituent through this bond by an addition reaction, then a second bond may be activated. If phenanthrene is bound through the 9:10 bond then the 1:2 bond would be available and possibly activated. Thus the formation of the 1:2-dihydroxy-1:2-dihydrophenanthrene is similar to the metabolism of other hydrocarbons which have been investigated.

The formation of the 9:10-dihydroxy-9:10-dihydrophenanthrene is anomalous as this process involves the addition of hydroxyl groups to the bond of high activity. In this reaction phenanthrene is metabolized in a manner unlike that occurring with the carcinogenic hydrocarbons.

Although *trans*-9:10-dihydroxy-9:10-dihydrophenanthrene can be prepared by reduction of the corresponding quinone this is unlikely to be the mechanism of the biological production of the diol because no diol could be isolated from the urine when phenanthraquinone was injected into rats or rabbits.

The fact that the hydroxyl groups in the metabolic diols obtained from naphthalene, anthracene (Booth & Boyland, 1949) and phenanthrene have the *trans* configuration suggests that they may arise by hydration of an epoxide. The bond to be oxidized may become activated by attachment of another part of the molecule to the enzyme system by an addition reaction. The activated bond might then react with an oxidizing system to yield an epoxide which might then react with water (by enzyme action in the cases where optically active diols are formed), with a tissue to form a complex or with an acid to yield an ester. The reaction of phenanthrene-9:10-epoxide with water might lead to formation of a racemic product, because the molecule is symmetrical about the reacting group. This and other possible mechanisms of the reaction of perhydroxylation are being investigated.

SUMMARY

1. When phenanthrene is injected into rats, *trans*-9:10-dihydroxy-9:10-dihydrophenanthrene is excreted in the urine; when injected into rabbits a mixture of the same diol and *laevo-trans*-1:2-dihydroxy-1:2-dihydrophenanthrene is excreted.

2. The oxidation of the 9:10-dihydroxy-9:10-dihydrophenanthrene with lead tetraacetate is unusual in that the *trans* compound reacts more rapidly than the *cis* form.

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A Study of the Pigments of the Sea-urchins, *Echinus esculentus* L. and *Paracentrotus lividus* Lamarck

By T. W. GOODWIN and S. SRISUKH
Department of Biochemistry, The University of Liverpool

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Although the purple pigments of the test (shell) and spines of the common sea-urchin *Echinus esculentus* L. have attracted some attention they have not been unequivocally identified. In his pioneer investigations MacMunn (1885) noticed a red quinonoid pigment which he named echinochrome in the perivisceral fluid of a number of sea-urchins; it was first isolated in crystalline form by McClendon (1912) from the ovaries of *Arbacia pustulosa* Leske, but it was not until much later that its empirical formula was established as $C_{12}H_{10}O_7$ (Ball, 1936; Glaser & Lederer, 1939; Tyler, 1939). Kuhn & Wallenfels (1939, 1940) isolated the same pigment, together with small amounts of two similar pigments, from the gonads of *Paracentrotus lividus* Lamarck and decided that it was 2-ethyl-3:5:6:7:8-pentahydroxy-1:4-naphthaquinone (I). Confirmation of this structure was obtained when Wallenfels & Gauhe (1943) achieved a synthesis of the pigment. Meanwhile, Lederer & Glaser (1938) isolated a new quinonoid pigment (spinochrome, spinochrome A, spinochrome P) from the spines and tests of the purple variety of *P. lividus*; this was confirmed by Musajo & Minchilli (1940), who later isolated a further green pigment spinochrome P_1 from the spines of the green variety of *P. lividus*.

Apart from MacMunn's (1885) work, the only other reports on the pigments of *Echinus esculentus* indicated that reduced echinochrome exists in the elaeocytes and that echinochrome itself is the pigment of the shell, dermis and spines (Lederer, 1940).

It was because of the paucity of information regarding the pigments of *E. esculentus* that the present investigation was undertaken. As the investigation proceeded it became apparent that two pigments were present in the tests and spines and that they were in fact spinochrome A (P) and spinochrome B (P_1); no evidence for the presence of echinochrome was ever obtained. Authentic samples of these two pigments were obtained from *Paracentrotus lividus* and the opportunity was taken not only of recording their properties more fully than had been done previously, but also of determining their distribution in the olive-green and violet varieties of this urchin.

Considerable confusion exists in the literature of sea-urchin pigments for two reasons: (i) various names have been given at different times to the same pigment, and (ii) authors have often been unaware of the synonomy existing in the systematic naming of sea-urchins. In order to clarify the position, Table 1, recording all the published information together with indications of synonomy in both chemical and systematic nomenclatures, has been compiled; names to be used in this paper are also indicated. The logical procedure has been adopted of identifying the pigments by adding the suffixes A, B, C, etc. after the general terms echinochrome and spinochrome, according to the order in which the pigments were isolated; this follows the procedure initiated by Kuhn & Wallenfels (1939). Much less difficulty arises in this way than in adding a suffix indicating the species from which the pigment was first isolated. In this way difficulties soon arise, e.g. spinochrome 'P' (*Paracentrotus*), has now been found in *Echinus esculentus*. The two group terms echinochrome and spinochrome have been accepted, for there appears to be good reason to believe that echinochromes occur only in the gonads and body fluid, whilst the spinochromes are characteristic of the spines and tests. The pigments obtained from Japanese sea-urchins (Kuroda & Oshima, 1940) are

Table 1. *Naphthaquinone pigments of sea-urchins*

Pigment		Sources				
Name used in this paper	Other reported names	Usual name	Common synonyms	Organs in which pigment is found	M.p. of pigment (°)	Reference
Echinochrome A	Echinochrome Arbacine	*Arbacia pustulosa* Leske	*A. aequituberculata* Blainville	Perivisceral fluid	—	McMunn (1885)
			A. lixula L.	Eggs, test, elaeocytes	—	McClendon (1912)
				Eggs, shell	—	Cannan (1927)
				Eggs	—	Vlés & Vellinger (1928)
				Eggs	220·5–221	Ball (1936)
				Ovaries	220	Lederer & Glaser (1938), Glaser & Lederer (1939)
				Ovaries	217	Kuhn & Wallenfels (1939)
		Echinus esculentus L.	—	Perivisceral fluid	—	McMunn (1885)
				Dermis, shell*	—	—
				Spines*	—	Lederer (1940)
		Paracentrotus lividus Lamarck	*Strongylocentrotus lividus* Lamarck, Brandt	Spines*	—	Glaser & Lederer (1939)
Echinochrome B	Echinochrome B	*Arbacia pustulosa*	As above	Ovaries	173–175	Kuhn & Wallenfels (1940)
Echinochrome C	Echinochrome C	*Arbacia pustulosa*	As above	Ovaries	—	Kuhn & Wallenfels (1940)
Echinochrome D	Isoechinochrome	*Arbacia pustolosa*	As above	Ovaries	247	Glaser & Lederer (1939)
Spinochrome A	Spinochrome	*Arbacia pustulosa*	As above	Spines and tests	185	Glaser & Lederer (1939)
	Spinochrome P	—	—	—	187–188	Musajo & Minchilli (1940)
					187–189	Tyler (1939)
	Echinorubin	—	—	—	—	Stern, quoted by Moore (1937)
		Echinus esculentus	—	Spines and tests	185 (sublimes)	The present work
Spinochrome B	Spinochrome P_1	*Arbacia pustulosa*	As above	Spines and tests	350–355	Musajo & Minchilli (1942)
		Echinus esculentus	—	Spines and tests	283 (decomp.)	The present work
	Spinochrome AKa	*Pseudocentrotus depressus*	—	Spines and tests	285–295	Kuroda & Oshima (1940)
	Spinochrome F	*Heterocentrotus mammilates*	—	Spines and tests	229	Kuroda & Oshima (1940)
	Spinochrome M	*Anthacidaris crassispina*	—	Spines and tests	193	Kuroda & Oshima (1940)

* Not confirmed in the present investigation.

not considered at the moment because of doubt concerning their possible identity with pigments previously described.

EXPERIMENTAL

Isolation of spinochromes A and B. A mixture of roughly broken tests and spines was first well washed with water, acetone and ethanol and then dissolved in 2N-HCl. The pigments were extracted into freshly redistilled ether which was then washed with small amounts of water until all the acid was removed. Care had to be taken not to overwash because, in the absence of acid, water extracts considerable amounts of pigment from the ether phase. The ethereal solution was dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo* at room temperature. The residue was dissolved in a small volume of dry redistilled ether and the ruby-red solution chromatographed on a $CaCO_3$ (British Drug Houses Ltd., A.R.) column. On eluting with ether five fractions were obtained: (*a*) a violet zone (most strongly adsorbed), (*b*) a pink zone, (*c*) an olive-green zone, (*d*) a yellow zone and (*e*) a yellow filtrate. Similar chromatograms were obtained with both *Echinus esculentus* and *Paracentrotus lividus* extracts although the relative amounts of (*a*) and (*c*) varied. The pigments (*b*), (*d*) and (*e*) were extremely minor constituents and were given only a cursory examination; little information could be obtained concerning (*d*) and (*e*), but (*b*) was characterized to some extent; (*d*) and (*e*) appear to be neither carotenoids nor naphthoquinones.

The developed column was extruded and the green, pink and violet zones separated and dissolved in N-HCl. The green and violet fractions were transferred to ether as previously described, and rechromatographed to remove final traces of impurities. After transferring the purified pigments to ether, the ether was removed and the pigments recrystallized three times from methanol. It should be noted that the olive-green pigment was obtained crystalline only from *P. lividus*; it occurs in *Echinus esculentus* only in traces.

The ruby-red pigment, which is violet on the column owing to the formation of a Ca salt, crystallized as fine dark violet rods, m.p. 185° (Found: C, 51·5; H, 3·5. Calc. for $C_{12}H_{16}O_3$: C, 51·1; H, 3·5%) and appears to be identical with the pigment isolated by Glaser & Lederer (1939), and by Musajo & Minchilli (1940), and termed spinochrome and spinochrome P respectively; as explained earlier, we consider spinochrome A a more appropriate name.

Table 2. *Properties of spinochromes A and B, and pigment X*

Reagents, etc.	Spinochrome A	Spinochrome B	Pigment X
Solubility			
Light petroleum (40–60°)	Insoluble	Insoluble	Insoluble
Ethyl ether	Very soluble	Very soluble	Very soluble
Ethanol	Very soluble	Very soluble	Very soluble
Methanol	Very soluble	Very soluble	Very soluble
Water	Very soluble	Soluble	Soluble
Chloroform	—	—	Soluble
Carbon disulphide	Very sparingly soluble (in hot)	Slightly soluble in warm, insoluble in cold	Soluble
n-Hexane	Very sparingly soluble (in hot)	Slightly soluble in warm, insoluble in cold	Soluble
Conc. H_2SO_4	Very soluble	Soluble	Soluble
NaOH (0·02N)	Very soluble soon decolorized	—	Black ppt.
Fluorescence	Nil in Et_2O solution	Nil in ethyl ether solution	—
Ferric chloride	Dirty green	Dirty green	Purplish Black ppt.
Methanolic lead acetate	Dark blue ppt. with blue solution	Brown ppt.	Brownish Black ppt.
Hydrogen peroxide	No action	No reaction	—
Optical activity	None demonstrable with concn. of 0·014 and 0·03% using 10 cm. cells	—	—
Mol. wt.	Too highly coloured in camphor for determination by Rast method	Insoluble in camphor	—

Table 3. *Absorption maxima of spinochromes A and B and pigment X in various solvents*

~ Denotes an inflexion.

Solvent	$\lambda\lambda_{max.}$ (mμ.) Spinochrome A	Spinochrome B	Pigment X
Ethanol	271, 310, 518	272, 320, 388, 480	267, 323, 468, ~520
Ethyl ether	254, 318, 525	272, 321, 388, 480	266, 323, 467, ~482, ~520
Chloroform	— — ~515, 530, ~572	— — 397, 474	— — 468, 488, ~522
Benzene	— — ~515, 540, ~582	— — 395, 478	— — 468, 488, ~524
Conc. H_2SO_4	262, 316, 495, 530, 565	276, 360, 445, 582	— — 440, 468, 500
n-Hexane	252, 316, 510, 543, 586	— — — —	252, 326, 460, 484, ~520
Carbon disulphide	— — 518, 550, 592	— — — —	— — 469, 484, ~527

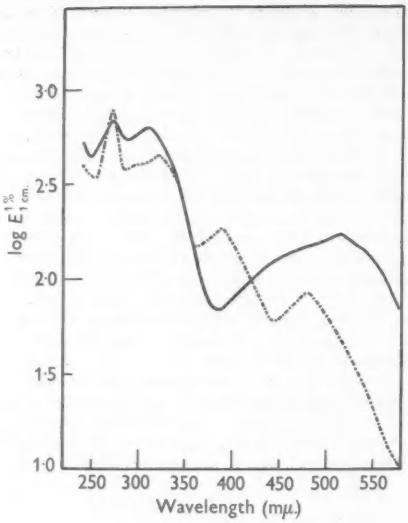


Fig. 1.

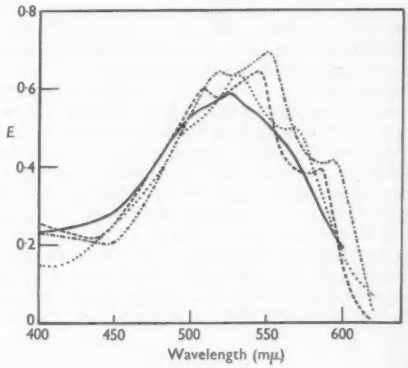


Fig. 2.

Fig. 1. The absorption spectra of spinochromes A and B in ethanol. ———, spinochome A; –·–·–·–, spinochrome B.

Fig. 2. The visible absorption spectrum of spinochrome A in various solvents. ———, in ether; – – – – – –, in *n*-hexane; –·–·–·–, in carbon disulphide; ·····, in conc. H_2SO_4.

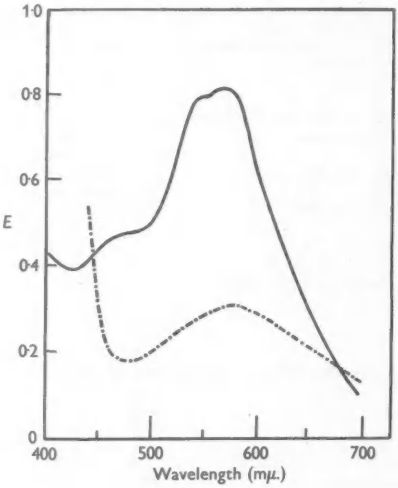


Fig. 3.

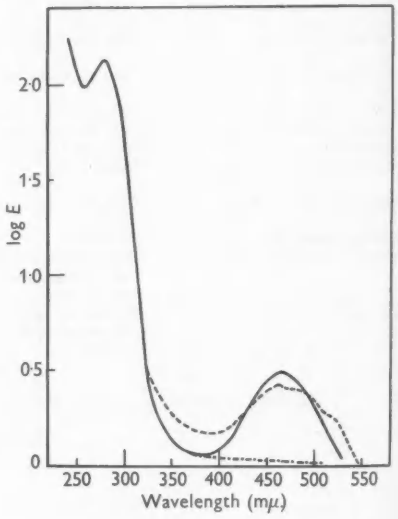


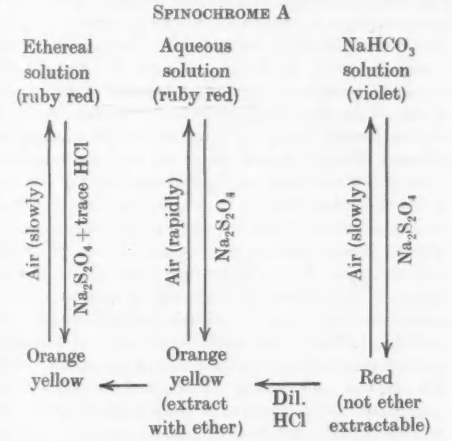
Fig. 4.

Fig. 3. The absorption spectra of spinochromes A and B in sat. $NaHCO_3$. ———, spinochrome A; –·–·–·–, spinochrome B.

Fig. 4. The absorption spectra of reduced spinochromes A and B and of pigment X. ———, spinochrome A; –·–·–·–, spinochrome B; – – – – – – –, pigment X.

The orange-red pigment, appearing on the column as an olive-green Ca salt, was isolated in crystalline form only from *Paracentrotus lividus*, but was unequivocally identified by spectroscopic and chemical tests in *Echinus esculentus*. The pigment (spinochrome B) crystallized as fine glistening thin red rods, m.p. 283° (decomp.) (Found: C, 51·3; H, 3·9. Calc. for $C_{12}H_{12}O_8$, C: 51·1; H, 3·9%). It is highly likely, from its absorption spectrum and general chemical properties, that it is identical with Musajo & Minchilli's (1942) spinochrome P_1, although these authors reported a m.p. of 350–355° (decomp.).

The general properties of these two pigments are recorded in Table 2. Previously the absorption spectra of these compounds have been recorded only in the visible spectral region, and in the case of spinochrome B (P_1) the solvent used was not specified. The complete spectra of these two pigments in ether are recorded in Fig. 1 and the absorption maxima in a number of other solvents are collected in Table 3. As the shape as well as the position of the visible band of spinochrome P is altered according to the solvent, the spectrum of this band in a number of solvents is recorded in Fig. 2; spinochrome B shows no such differences in shape. The spectra of the two pigments in alkaline solution and when reduced are also recorded (Figs. 3, 4). Being quinonoid derivatives the pigments are easily reducible with dithionite and equally rapidly reoxidized in air. By working very quickly in ethereal solution it was, however, possible to measure the spectra of the reduced forms. The reduction of spinochrome A follows different paths depending on whether the reaction takes place in acid or alkaline solution, viz.



By contrast, a solution of spinochrome B in $NaHCO_3$ which is olive green is reduced to a light-yellow solution by $Na_2S_2O_4$ which, on acidification, is immediately oxidized to its original orange-red form.

The pigment from the pink zone (pigment X) was transferred to ether in the usual manner, but, although it was purified sufficiently to be completely free from any traces of the two other pigments, insufficient was obtained to make crystallization feasible. However, its spectrum was obtained in a number of solvents (Figs, 4, 5; Table 3), and its general properties examined (Table 2). They were very similar to spinochrome A, and it is undoubtedly a naphthaquinone type of compound.

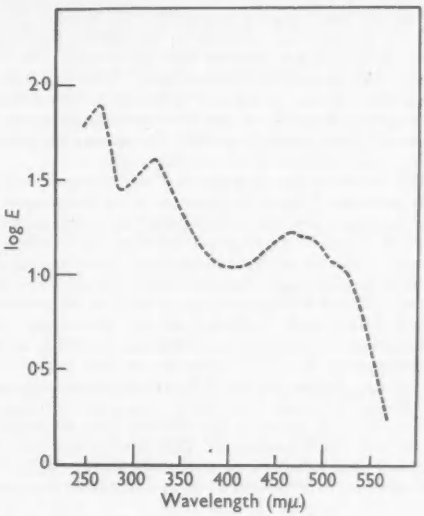


Fig. 5. The absorption spectrum of pigment X in ether.

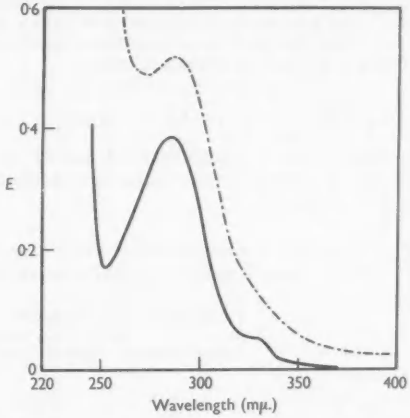


Fig. 6. The absorption spectra of the leuco acetyl derivatives of spinochromes A and B. ———, leuco acetyl derivative of spinochrome A; –·–·–, leuco acetyl derivative of spinochrome B.

Reductive acetylation of the pigments. The pigments were reductively acetylated in a conventional manner: 10 mg. of crystalline pigment in 2 ml. of glacial acetic acid were refluxed for 1 hr. on a water bath with 1 g. of Zn dust and 0·5 g. of fused Na acetate. In the case of spinochrome A the colour changed from ruby red to yellow almost immediately

but no further colour changes took place; with spinochrome B, however, the orange-red colour was completely discharged. When the reaction was complete the resulting mixture was filtered into ice water and allowed to stand overnight, when a granular precipitate was obtained. In the case of spinochrome A efforts to recrystallize the small amount of material available were unsuccessful, but the leuco acetyl derivative of spinochrome B was crystallized from warm glacial acetic acid as colourless fine rod-like crystals which melted at 226–230° (decomp.); Musajo & Minchilli (1942) report m.p. 235°. The spectra of the two leuco acetyl derivatives are recorded in Fig. 6.

Determination of the distribution of spinochromes A and B. The amounts of these two pigments in various samples of test and spines were determined using a spectrophotometric method based on a procedure described by Goodwin & Morton (1946) for the determination of tyrosine and tryptophan in proteins; this consists of measuring the absorption at two selected wavelengths, one of which is the isosbestic point of the system. In the present case when ether is the solvent the isosbestic point is at 435 mμ. and $E^{1\%}_{1\,cm.}$ at this wavelength is 65 ($E^{1\%}_{1\,cm.}$ values can be used instead of ϵ values because the mol.wt. of the two pigments are almost identical); the other wavelength chosen was 525 mμ. at which the $E^{1\%}_{1\,cm.}$ values for spinochrome A and spinochrome B are 149 and 78 respectively. Thus, after determining the total pigment concentration from the E 435 mμ. value, the composition of a mixture can be calculated using the equation $y=\frac{E-xB}{A-B}$, where y = concn. of spinochrome A, x = total pigment concn., $A=E^{1\%}_{1\,cm.}$ 525 mμ. value for pure spinochrome A, $B=E^{1\%}_{1\,cm.}$ 525 mμ. value for pure spinochrome B and E is the observed extinction at 525 mμ. The presence of the other pigments can have little or no effect on the results as they are present only in traces.

RESULTS AND DISCUSSION

The distribution of spinochromes A and B in *E. esculentus* and *Paracentrotus lividus* are recorded in Table 4.

Table 4. *Amounts of spinochromes A and B in spines and tests of* Echinus esculentus *and* Paracentrotus lividus

Material	Amount (mg./g. fresh wt.) Spinochrome A	Spinochrome B
E. esculentus		
Spines (violet)	0·0019	Trace
Shell (violet)	0·023	Trace
P. lividus		
Spines (violet)	0·52 0·50	0·39
Spines (olive green)	0·17	1·07

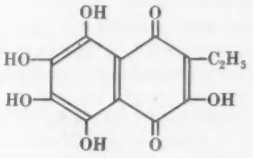
This work has demonstrated by isolation of the material in crystalline form that spinochrome A is the major pigment occurring in the spines and tests of *Echinus esculentus*; this does not confirm Lederer's (1940) short report that the pigment is echinochrome, but does verify Stern's unpublished observations (see Moore, 1937) that the pigment was slightly different from echinochrome. Spinochrome B, the orange-red component of the pigment system of *Paracentrotus lividus* spines was also identified in *Echinus esculentus*, but was not present in sufficient amounts for isolation.

The spinochrome A isolated from *Paracentrotus lividus* is identical with that isolated by Glaser & Lederer (1939) and Musajo & Minchilli (1940); spinochrome B is identical with spinochrome P_1 isolated from olive-green *P. lividus* by Musajo & Minchilli (1942) in all properties except melting point, but as the melting points of the leuco acetyl derivatives prepared by Musajo & Minchilli (1942) and ourselves are almost identical, it is reasonable to assume that the pigments are in fact identical.

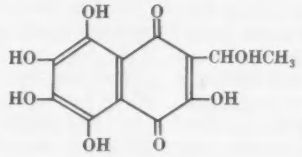
The violet and olive-green colours encountered in the spines are due to the formation of salts between the pigments and the calcium of the test and spines, the normal colour of the pigments being ruby red and orange red respectively. The formation of such salts is readily seen on chromatography of the pigments on calcium carbonate when they separate into bright violet and olive-green bands; even brighter colours are produced on magnesium carbonate. It is important to correct an impression given by the work of Musajo & Minchilli (1940, 1942) that in the case of *P. lividus*, violet specimens contain only spinochrome B, whilst olive-green specimens contain both spinochromes A and B. The fact that both pigments exist in both variants is immediately apparent on chromatography of a crude extract, and is also apparent if the spines from the two varieties are examined closely. The spines of the olive-green animals always have a violet tip and in occasional cases this violet area can extend one-third of the way down the spine. In the violet animals the reverse is the case, small areas of olive-green pigments are always present at the base of the spines, while the rest is violet. The observation that the difference between the coloration of the violet and olive-green specimens is merely quantitative is well brought out in Table 4. There is apparently no clear-cut biological differentiation between the two colour variants of *P. lividus*, but considering the relative positions of the pigments in the species, it may well be that they represent different developmental stages. It is also important to realize that Glaser & Lederer's (1939) suggestion that the olive-green coloration is due to a mixture of echinochrome A and spinochrome A cannot be correct.

Structures of spinochromes A and B. Spinochrome A is very similar in its properties to echinochrome A (I) and must differ only slightly from it in structure. The analytical results of Lederer & Glaser (1938) and of ourselves suggest that it contains an additional hydroxyl group compared with echinochrome A;

1 mole of acetic acid is obtained on oxidizing 1 mole of the pigment (Lederer & Glaser, 1938; Musajo & Minchilli, 1940) which is consistent with a —CHOH.CH_3 side chain. The spectroscopic data are consistent with the replacement of ethyl by —CHOH.CH_3 in position 2, for considerable work (e.g. see Spruit, 1949) has indicated that the introduction of varying saturated side chains into naphthaquinones has little effect on their absorption spectra and the visible spectrum of echinochrome A



(I)



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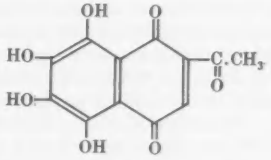
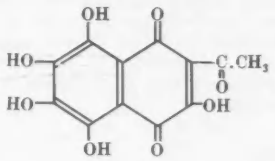


(III)

is, in position at least, very little different from that of spinochrome A; further, it should be noted that substitution of ethyl by methyl has no effect on the spectrum of echinochrome (Weygand, Vogelbach & Zimmerman, 1947) and that the complete spectrum of solanione (2-methyl-3-methoxy-6-(or 7-)-acetonyl-5:8-dihydroxy-1:4-naphthaquinone) is very similar to that of spinochrome A (Weiss & Nord, 1949). According to Kuhn & Wallenfels (1941) the hydroxyl group is present or absent in position 3 (Spruit, 1949).

Musajo & Minchilli (1942) consider that (III) represents the structure of spinochrome B; there are a number of reasons why this cannot be accepted; viz. (*a*) our analytical data cannot be reconciled with this structure; (*b*) the introduction of an acetyl group into position 2 of a naphthaquinone has little if any effect on its spectrum (Spruit, 1949) and thus the spectrum of spinochrome B should be very similar to that of spinochrome A; (*c*) spinochrome B exhibits an absorption band at 388 mμ. (not noted by Musajo & Minchilli (1942) who did not record its spectrum below 400 mμ.) which is atypical of a simple hydroxy-substituted naphthaquinone (Spruit 1949); and (*d*) Musajo & Minchilli (1940, 1942) obtain very much less acetic acid on oxidation of the pigment than on oxidation of spinochrome A; according to Kuhn & Wallenfels's (1941) work on



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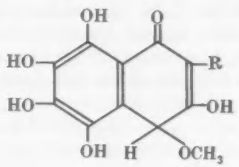
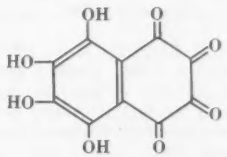
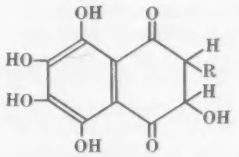


(V)



(VI)



(VII)

presence of an enolizable hydroxyl group in the side chain can account for the difference between spinochrome A and echinochrome. It seems reasonably certain then that the structure of spinochrome A (II) suggested by Kuhn & Wallenfels (1941) is correct and that the analytical data cannot be reconciled with Musajo & Minchilli's (1940) suggestion that no hydroxyl is present in position 3; it should be noted, however, that it is virtually impossible to decide from spectroscopic data alone whether an spinon A (IV) the same amount of acetic acid should have been obtained in each case.

Any structure suggested for spinochrome B must attempt to explain, at least qualitatively, its atypical spectrum. The two most plausible structural types are (*a*) a hemiquinone and (*b*) a dihydroquinone. The pigment cannot be an ordinary hemiquinone because it ought to be easily oxidizable in air or by hydrogen peroxide to a pigment similar to spinochrome A; as this does not occur it is suggested that

the hydroxyl in position 4 may be methylated and thus the system is stabilized in a structure such as (V). Such a compound has been produced from benzoquinone by the action of Grignard reagent (e.g. see Karrer, 1938). Regarding the second possibility, Kuhn & Wallenfels (1942) produced by the action of silver oxide on echinochrome A, a substance dihydroechinochrome (VI) which exhibits a band at 380–390 mμ. and an inflexion at about 420 mμ. The analytical data rule out such a structure for spinochrome B, but (VII) may well be correct. It should be emphasized that we are here mainly concerned with suggesting the type of structure spinochrome B may possess; considerably more data are required before a complete structure can be suggested; lack of material has limited our investigations along these lines. The spectrum of the reduced form of spinochrome B is also consistent with a structure of the type (V) or (VII) for it displays only one band at 290 mμ. (Fig. 3). This band can be ascribed to absorption by the hydroxybenzenoid nucleus and there is no visible absorption band due to naphthol absorption (Spruit, 1949) because no such residue exists. This is in contrast to the spectrum of reduced spinochrome A which exhibits absorption bands characteristic of both hydroxybenzenes and naphthols.

The pigment which forms a pink calcium salt (pigment X) must be a naphthaquinone pigment because it exhibits very similar properties to spinochromes A and B (see Table 2); its absorption spectrum indicates that it is more closely allied to spinochrome A than to spinochrome B. We have refrained from naming it because it has not been isolated crystalline.

SUMMARY

1. Five pigments have been detected in the sea-urchins *Echinus esculentus* and *Paracentrotus lividus*; of these three are naphthaquinone pigments and two are neither naphthaquinones nor carotenoids.

2. The two major pigments spinochromes A (P) and B (P_1) are naphthaquinone derivatives and have been isolated and their properties, especially spectral, studied in detail.

3. The properties of the third naphthaquinone pigment which has not been isolated are reported.

4. A spectroscopic method for the determination of the amounts of spinochromes A and B in a mixture of the two is described.

5. The distribution of the two major pigments in the spines and tests of *Echinus esculentus* and the olive-green and violet variants of *Paracentrotus lividus* has been examined.

6. The data obtained are consistent with the structure suggested by Kuhn & Wallenfels (1939) for spinochrome A, but not with that ascribed by Musajo & Minchilli (1942) to spinochrome B. Possible structures of spinochrome B are discussed.

We are grateful to Mr J. R. Bruce, Marine Biological Station, Port Erin, I.O.M., and Prof. L. P. Renouf, University College, Cork, for samples of *Echinus esculentus* and *Paracentrotus lividus* respectively. Mr Bruce was also extremely helpful regarding the nomenclature of sea-urchins. Thanks are also due to Sir E. Mellanby, F.R.S., who, through the British Council, obtained for us Italian journals not available in this country. The encouragement and guidance of Prof. R. A. Morton, F.R.S., is gratefully acknowledged.

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Antithyroid Substances

1. THE MERCAPTOGLYOXALINES

By C. E. SEARLE, A. LAWSON AND A. W. HEMMINGS
University College Hospital Medical School and Royal Free Hospital School of Medicine, London

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The high antithyroid activity of 2-mercaptoglyoxaline and its 4-ethyl and 4-propyl derivatives has been reported by Astwood, Bissell & Hughes (1945), and Jackman, Klenk, Fishburn, Tullar & Archer (1948). Moreover, 2-mercaptoglyoxaline has been shown by Stanley & Astwood (1947) to be among the most potent antithyroid compounds tested on man. Similar results, obtained by us with rats in an investigation of substances related to ergothioneine, led us to undertake a more systematic study of compounds possessing this ring system. The mercaptoglyoxaline structure (Fig. 1) seems to be particularly suitable

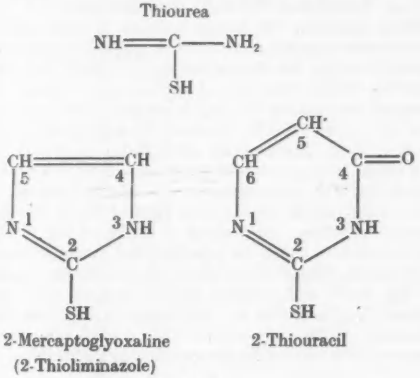

Fig. 1.

for the investigation of the effect of substituents on the antithyroid activity of thiol compounds because it offers a simpler ring system than that in the thiouracil series where there is a strongly polar carbonyl group.

Various workers have suggested that the antithyroid effect of thiol drugs is dependent on interference with an oxidative system in the thyroid gland responsible for the conversion of iodide to iodine, this interference being regarded as due to the reducing action of the thiol group. All the mercaptoglyoxalines showing appreciable activity have been found to react with iodine in the cold, and the oxidation of a number of thiols to the corresponding disulphides has been described by Biltz & Krebs (1912) and Balaban & King (1927; cf. also Miller, Roblin & Astwood, 1945). On this hypothesis, having in mind the benzenoid character of the glyoxaline ring, one would expect that an important factor influencing the potency of compounds of this type would be the ease of ionization or the lability of the hydrogen atom attached to the sulphur, and that such lability would be facilitated when electron-attracting groups were present or when the structure of the anion was such that it had a high resonance stability.

Such a hypothesis, however, is far from being adequate to account for all the facts. In the cases of aliphatic thiol compounds such as cysteine and glutathione, which are without antithyroid action, it may be that the great ease with which these undergo oxidation to disulphide forms does not allow them to reach the gland in a reduced state. Again, in the cases of 4:5-diphenyl-2-mercaptoglyoxaline (Astwood *et al.* 1945) and 2-mercaptonaphthiminazole (Bywater, McGinty & Jenesel, 1945) which have very low potency as compared with 2-mercaptobenziminazole, other factors must obviously operate. On the basis of the results described in this communication it is difficult to show any consistent correlation between the electronic character of the substituent groups and the antithyroid potency of the mercaptoglyoxalines investigated.

Of the various methods which have been used for the assay of antithyroid substances, those employing radioactive iodine (^{131}I) as a labelling agent for estimating interference with iodine uptake and concentration by the gland are probably the most convenient. Rats have been employed satisfactorily by several workers (Rawson, Tannheimer & Peacock, 1944; Franklin, Lerner & Chaikoff, 1944). Rawson, McGinty, Peacock, Merrill, Wilson & Lockhart (1948) demonstrated the blocking effect upon radioactive iodine uptake of antithyroid drugs administered over several days, whilst Larson, Keating, Peacock & Rawson (1945) similarly demonstrated the effectiveness in chicks of a single subcutaneous injection of 10 mg. of thiouracil. They further showed that the relation between the degree of inhibition and the logarithm of the dose of thiouracil was a linear function. McGinty, Rawson, Fluharty, Wilson, Riddell & Yee (1948) compared a number of anti-

thyroid drugs by giving rats or chicks a single dose by injection followed 1 hr. later by an injection of radio-iodine. After 4 hr. the animals were killed and the uptake of isotope by their thyroid glands compared with that of controls. It was clearly established that within 8 hr. after administration, partial recovery of the gland from the action of the drug took place in all cases.

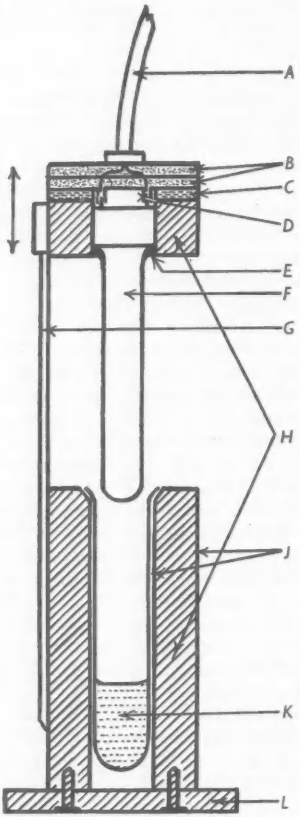

Fig. 2. Castle and mounting for Geiger-Müller immersion type counter. *A*, coaxial cable to scaling unit; *B*, 'Paxolin' caps; *C*, rubber washer; *D*, contacts; *E*, paraffin wax; *F*, Geiger-Müller counter; *G*, angle-brass guide; *H*, lead shielding; *J*, brass tubing; *K*, radioactive solution; *L*, removable lead base.

In the present work we have used a modification of the method of McGinty *et al.* (1948). This technique affords a rapid screening method by which promising drugs may be picked out for further study. It was necessary to ascertain whether or not the linear log. dose:response relationship exhibited by thiouracil was applicable also to the series of compounds under investigation, and for this purpose 2-mercaptoglyoxaline was selected as a representative compound for study.

METHODS

The synthesis of new compounds used in this investigation will be described elsewhere.

Test animals. Black and white hooded rats weighing approximately 50 g. were maintained for a minimum of 2 weeks, before testing, upon a batch of commercial cube diet known to be somewhat low in I_2 content (0·16–0·28 mg./kg.), but not so low as to produce goitrous animals. With diets of higher I_2 content, the uptake of ^{131}I was inconveniently low and less useful for the detection of compounds of low potency. Other batches of the same commercial diet have been shown to contain three to four times the above quantity of I_2, due, we suspect, to differences in the quality and origin of the fish-meal component. Animals were used in groups of five to eight for each assay; in a few instances where experiments have been carried out more than once, the results have been combined.

Dosing. A single dose of drug in aqueous solution was administered by stomach tube, in most cases at the level of 5 or 10 mg./kg. body wt.; when solubility difficulties were encountered, the Na salts or hydrochlorides were used.

Isotopic iodide. The stock of isotopic I_2 solution was diluted with 0·9% (w/v) NaCl to approximately 1·0 μc. of ^{131}I/ml. No addition of carrier iodide was made.

Assay technique. The isotope solution (0·5 ml.) was injected intraperitoneally into each rat 1 hr. after administration of the drug, and after a further 4 hr. the animals were killed by $CHCl_3$. The thyroid gland of each animal was dissected out, placed in a pyrex boiling tube containing 2 ml. of 2% (w/v) NaOH, dissolved by warming and diluted to about 15 ml. For counting, each solution was transferred to a single boiling tube suitably graduated for the relative dimensions of the tube and immersion counter, and made up to the mark (about 18 ml.) with distilled water. The lead 'castle' containing the counter is shown in Fig. 2. The solution (0·5 ml.) used for injection was similarly treated with NaOH, diluted and counted. The percentage uptakes of the dosed and undosed control animals were then compared. The results were expressed as the percentage depression of the mean uptake of the control animals, the significance of the results being evaluated statistically.

RESULTS AND DISCUSSION

The variation of ^{131}I uptake with time is illustrated by Fig. 3. Notwithstanding the larger uptake obtained over 24 hr., an interval of 4 hr. was selected for the single-dose experiments, since considerable lessening of drug action has been shown to occur within 8 hr. under similar circumstances by Larson *et al.* (1945). In experiments where drugs are continuously administered in the diet over several days, we prefer to use a 24 hr. period of ^{131}I uptake.

The dose-response curve of 2-mercaptoglyoxaline was found to be very similar to that of 2-thiouracil, although slightly more steep (Fig. 4). Both are typically S-shaped curves in which the central portion approximates to a straight line (cf. Larson

et al. 1945). It follows from this that valid comparisons of potency are best made between the limits of 20 and 80% inhibition of iodine uptake and also that comparisons with thiouracil should be drawn with caution.

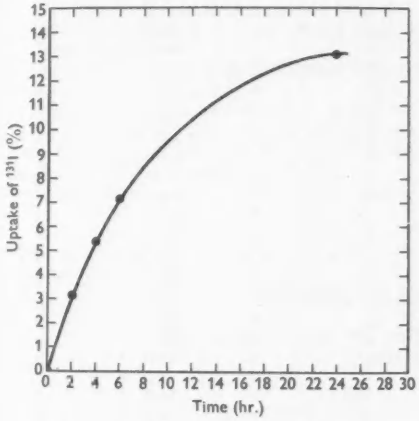

Fig. 3. Variation of ^{131}I uptake with time.

2-Thiolhistidine, its methyl betaine (ergothioneine), and 4-methyl-2-thiolhistidine in this test showed no significant activity. This result for ergothioneine is in agreement with that of Stanley & Astwood (1947) and Wilson & McGinty (1949) who were unable to confirm the finding of Lawson & Rimington (1947). Further work is proceeding with this substance.

For purposes of comparison we have included amongst the compounds examined potassium thiocyanate, thiourea, thiouracil and its 6-amino, 6-methyl, and 6-propyl derivatives. The results for these compounds, shown in Table 1, are in agreement with those reported by other workers using different methods of assay.

SUMMARY

A series of 2-mercaptoglyoxaline derivatives has been tested for antithyroid activity in rats using a single-dose technique combined with radioactive iodine uptake.

We wish to thank Glaxo Laboratories for their generosity in providing most of the rats used in the investigation, and the Medical Research Council Tracer Committee for the supply of radioactive iodide. We are indebted to Mr R. A. F. Bullerwell, Mr H. Heath, and Mr H. V. Morley, who prepared most of the compounds examined, and also to Sir Charles Harington, F.R.S., Burroughs Wellcome & Co., and the Lederle Laboratories for gifts of 2-thiolhistidine, ergothioneine and 6-aminothiouracil respectively. We are grateful to Miss B. E. Petchey for technical assistance and particularly to Prof. C. Rimington for continued interest and advice on the work. One of us (C. E. S.) acknowledges, with thanks, a grant from the Rockefeller Bequest to University College Hospital Medical School.

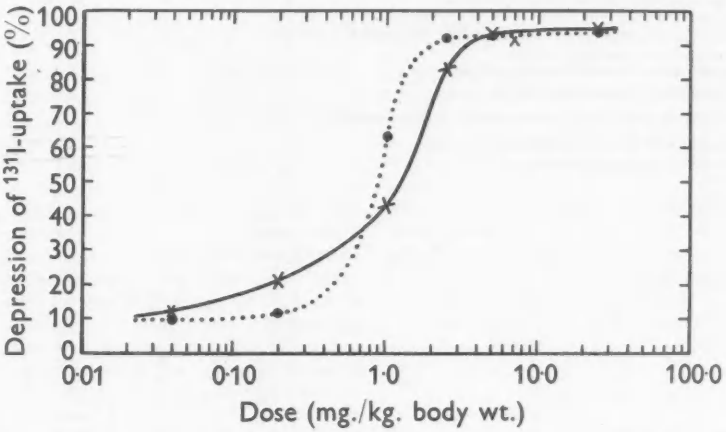

Fig. 4. Dose-response curves for 2-thiouracil (×—×—×) and 2-mercaptoglyoxaline (●···●···●). Single dose at 0 hr.; ^{131}I at 1 hr.; killed at 5 hr.

The mercaptoglyoxalines tested by the method described fall mainly into two groups, those in one group causing a depression of iodine uptake of under 30%, and those in the other of over 80% (Table 1). The parent compound and its 4-alkyl derivatives were all of high potency, although in this series, the introduction of the substituent alkyl group did not increase the activity as has been shown to occur with the thiouracils (Astwood *et al.* 1945).

Table 1. *Antithyroid activity of compounds as tested by the single injection technique in rats*

Drug	Dose (mg./kg.)	No. of rats dosed	Percentage depression of ^{131}I uptake	Significance of difference from control
2-Mercaptoglyoxaline	0·04	4	9·5	*C*
	0·20	5	11·7	*C*
	1·0	5	63·0	*A*
	2·5	6	92·4	*A*
	5	15	93·8	*A*
	25	11	94·7	*A*
4-Methyl-2-mercaptoglyoxaline	5	5	51·1	*B*
	6	6	61·1	*B*
4-Ethyl-2-mercaptoglyoxaline	5	4	87·7	*A*
	6·5	7	85·7	*A*
4-*n*-Propyl-2-mercaptoglyoxaline	5	5	86·8	*A*
4-*n*-Amyl-2-mercaptoglyoxaline	8·5	8	82·7	*A*
4:5-Dimethyl-2-mercaptoglyoxaline*	5	5	15·0	*C*
	6·5	7	−1·8	*C*
4-Methyl-5-ethyl-2-mercaptoglyoxaline	5	10	5·8	*C*
4-Ethyl-5-*n*-propyl-2-mercaptoglyoxaline	8·5	5	41·9	*A*
4-Phenyl-2-mercaptoglyoxaline	9	8	60·0	*A*
2-Mercaptobenziminazole	7·5	6	94·8	*A*
2-Mercapto-1′:2′-naphthiminazole	10	8	−21·8	*B*
3-Hydroxymethyl-2-mercaptoglyoxaline	5	5	94·2	*A*
	7	6	96·8	*A*
2-Benzylthioglyoxaline	10	5	26·4	*B*
4-Methyl-2-acetylmercaptoglyoxaline	8	6	57·7	*A*
4-Methyl-5-aldehydo-2-mercaptoglyoxaline	10	5	63·0	*A*
2-Mercaptoglyoxaline-4-carboxylic acid	7	7	9·7	*C*
4-Carbethoxy-2-mercaptoglyoxaline	8·5	7	70·5	*A*
4-Methyl-5-carbethoxy-2-mercaptoglyoxaline	5	5	2·6	*C*
4-*p*-Hydroxybenzyl-2-mercaptoglyoxaline	5	5	10·7	*C*
4-Methyl-5-*p*-hydroxybenzyl-2-mercaptoglyoxaline	5	4	12·2	*C*
4-($\alpha\beta\gamma\delta$-Tetrahydroxybutyl)-2-mercaptoglyoxaline	11	6	10·25	*C*
4-Aminomethyl-2-mercaptoglyoxaline	5	5	12·8	*C*
Product from action of formaldehyde on preceding compound	10	5	18·1	*B*
4-Carbethoxyamino-2-mercaptoglyoxaline	5	5	15·9	*B*
4-Dimethylaminomethyl-2-methylthioglyoxaline	10	5	7·9	*C*
3-Methyl-4-acetamido-2-mercaptoglyoxaline	5	5	15·9	*C*
4-Methyl-5-dimethylaminomethyl-2-mercaptoglyoxaline (acetate)	12	5	24·5	*B*
	50	5	27·7	*B*
3-Methyl-5-carbethoxy-4-amino-2-mercaptoglyoxaline	10	5	25·9	*C*
4-Methyl-5-bromo-2-mercaptoglyoxaline	10	6	15·1	*C*
2-Thiolhistidine	10	5	−7·5	*C*
4-Methyl-2-thiolhistidine	10	5	6·7	*C*
Ergothioneine (HCl, $2H_2O$)	0·04	5	6·3	*C*
	0·20	5	14·6	*C*
	1·0	16	8·9	*C*
	5	14	9·1	*C*
	15	5	7·1	*C*
	20	6	3·0	*C*
	25	16	−1·5	*C*
	75	6	14·8	*C*
2-Thiouracil	0·04	6	11·5	*C*
	0·20	6	21·0	*C*
	1·0	6	42·7	*B*
	2·5	6	83·5	*A*
	5	6	93·5	*A*
	7	5	91·4	*A*
	25	6	95·3	*A*
6-Methyl-2-thiouracil	7	6	92·6	*A*
6-*n*-Propyl-2-thiouracil	8·5	6	96·3	*A*
6-Aminothiouracil	7	10	−21·6	*B*
2-Thiohydantoin	6	6	89·8	*A*
1-Benzyl-2-thiohydantoin	10	5	88·2	*A*
Thiourea	4	5	41·0	*B*
Potassium thiocyanate	5	6	11·3	*C*

* This compound was also administered over a period of 9 days at a level 0·25% in the diet and showed a cumulative effect resulting in a depression of ^{131}I uptake of 43·0%.

The significance of differences in ^{131}I uptake between treated and control animals was calculated using Student's t test

$$\left[t = \frac{\text{Difference between } ^{131}\text{I uptake of dosed and control animals}}{\text{Estimated standard deviation}} \sqrt{\left(\frac{N_1 N_2}{N_1 + N_2}\right)}\right].$$

The level of significance is indicated in the last column of the table as follows: $P < 0{\cdot}001$, *A*; $0{\cdot}1 > P > 0{\cdot}001$, *B*; $P > 0{\cdot}1$, *C*.

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Studies in Congenital Porphyria

1. INCORPORATION OF ^{15}N INTO COPROPORPHYRIN, UROPORPHYRIN AND HIPPURIC ACID

By C. H. GRAY and A. NEUBERGER

Department of Chemical Pathology, King's College Hospital, Denmark Hill, London, S.E. 5, and the National Institute for Medical Research, Hampstead, London, N.W. 3

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Like other rare inborn errors of metabolism, congenital porphyria is of considerable genetic interest and might be expected to give valuable information about normal metabolism in man. Congenital porphyria has been the subject of a large number of investigations, but the reason why large amounts of highly carboxylated porphyrins of series I are formed and excreted has so far not been elucidated. Shemin & Rittenberg (1946) have shown that glycine is the specific precursor of at least some and probably all the nitrogen atoms of the protoporphyrin in circulating haemoglobin. Later work of Wittenberg & Shemin (1949) and Muir & Neuberger (1949) has shown that at least one of the nitrogen atoms of rings I and II and one of rings III and IV, respectively, are derived from glycine. It is therefore to be expected that, if the porphyrins of series I and III have part of their metabolic pathway in common, glycine should also be the precursor of the nitrogen atoms of coproporphyrin and uroporphyrin I. Owing to the fact that the labelled newly formed haem is greatly diluted with pre-existing unlabelled haem, the ^{15}N content of the newly formed protoporphyrin present in haemoglobin can only be calculated by a mathematical analysis (Shemin & Rittenberg, 1946). In a porphyric, on the other hand, we may assume that excreted porphyrins are not diluted to any large extent by material formed prior to the date of administration of labelled glycine and we might thus expect to get an approximate but direct measure of the ^{15}N content of newly formed porphyrins.

The present paper deals with the incorporation of ^{15}N into the porphyrins, into the haem of circulating haemoglobin and into the urinary hippuric acid after feeding with labelled glycine a patient suffering from congenital porphyria. We have also investigated the incorporation of ^{15}N into the haem of the circulating haemoglobin of a normal person given a similar amount of labelled glycine.

Some of the results reported here were the subject of a preliminary communication (Gray & Neuberger, 1949).

EXPERIMENTAL

General

Experiments with a porphyric

The subject was a 32-year-old male, G.L. (60 kg. body weight), who had suffered from porphyria since birth. His clinical history up till the age of 9 has been described by Mackay & Garrod (1926). Since then, he has continually excreted mahogany-coloured urine and the skin condition has progressed each summer. As a result of secondary infection he has now lost an eye, both pinnae of the ears and all the terminal phalanges of his hands. The skin of the exposed parts is a mass of scar tissue intermingled with patches of deeply pigmented skin. The patient was given labelled glycine in two separate experiments.

Experiment 1. The subject was put on a diet containing only 20 g. of animal protein/day, beginning 26 January 1949. On the morning of 30 January 1949 he was given the first of 12 doses of 1 g. of labelled glycine, which were given every 6 hr. during the following 3 days. The glycine contained 31·65 atom % excess ^{15}N. On 10 February 1949 all restriction on his diet was discontinued. The urine and faeces were collected separately in 24 hr. batches for the first 12 days after glycine feeding. From the 2nd to the 9th day and on the 12th day after glycine feeding, 3 g. of Na benzoate were given by mouth at 6 a.m. The urine excreted during the succeeding 4 hr. periods was collected separately from the remaining 20 hr. urine of the 24 hr. collection. These 4 hr. collections were used for the isolation of hippuric acid. Subsequently, faeces were collected and worked up for pigments in 4- or 6-day batches during the succeeding 160 days except for a few batches collected between the 40th and 60th day. An occasional 24 hr. collection of urine was also investigated during this period. Blood samples (usually 10 ml.) for preparation of haemin were taken at convenient intervals.

Experiment 2. On 4 September 1949 (that is 217 days after the beginning of the first glycine feeding) the patient was again put on a diet, this time containing 40 g. animal protein/day, and on 7 September 1949 he was given another 12 doses of 1 g. of glycine containing 31·68 atom % excess ^{15}N. This time the glycine was given every 2·5 hr. Urine was not collected on this occasion, but faeces passed from the 2nd to the 9th day after glycine feeding were collected and worked up for stercobilin. Blood samples for the isolation of haemin were taken first at 3-day, then at 4-day intervals, and 40 days after the glycine feeding at fortnightly intervals.

Experiment with normal male

A normal male subject (67·6 kg. body weight) was put on a diet containing only 20 g. protein/day beginning 14 April 1949. On the morning of 21 April he was given the first of 12 doses of 1 g. of labelled glycine which was given every 6 hr. during the next 3 days. The glycine contained 31·65 atom % excess ^{15}N. On 27 April the restriction on his diet was discontinued. Na benzoate (3 g.) was given by mouth at 8 a.m. on 25 April and an identical dose again at 11.15 a.m. on 27 April. The urine was collected for hippuric acid isolation in 4 hr. batches following each dose of Na benzoate. Blood samples were taken at suitable intervals and the protoporphyrin isolated as described below.

Chemical methods

Isolation of urinary porphyrins. The urine was acidified with 1/20 of its volume of glacial acetic acid and extracted with equal volumes of ether three times. The small amounts of emulsion were separated by centrifuging, the resulting interfacial precipitate and aqueous layer then being added to the ether-extracted urine. The coproporphyrin was transferred to 2·7 N-HCl, and after adjusting with NaOH and K acetate to pH 3·0–3·2, taken back into ether. The ether layer was washed with water containing a trace of K acetate, and evaporated to dryness. After esterification with methanolic HCl, the coproporphyrin tetramethyl ester was transferred to $CHCl_3$ in the usual way, and finally crystallized four times from hot $CHCl_3$-methanol. Typical crystals of coproporphyrin I tetramethyl ester m.p. 253–257° (corr.) were obtained.

The ether-extracted urine, to which all interfacial precipitates had been added, was allowed to stand for about 5 days during which time a deposit of impure uroporphyrin settled at the bottom of the vessel. The supernatant was siphoned off and separated from the crude uroporphyrin by centrifuging. This precipitate was then washed by stirring with 2% (w/v) acetic acid followed by centrifuging. The precipitate was repeatedly extracted with small quantities of 2·7 N-HCl until the extracts were no longer red in colour. K acetate-solution was added to the deep red solution until the pH was approximately 3·2. On standing, the uroporphyrin settled out practically quantitatively and was centrifuged off and dried in a vacuum desiccator over P_2O_5. The porphyrin was then esterified with methanolic HCl, and the resulting ester, after transfer to $CHCl_3$, recrystallized six times from hot $CHCl_3$-methanol. Typical crystals of uroporphyrin I octamethyl ester m.p. (corr.) 290–293° were obtained.

Isolation of faecal coproporphyrin. The faeces were ground in a mortar with about a quarter of their bulk of glacial acetic acid and then with an equal volume of ether. On standing for a short while, a deeply pigmented layer separated out and could be decanted through a large filter paper. The residue was repeatedly extracted with further quantities of ether, fresh acetic acid being added at about every fourth ether extraction, until the extracts were pale in colour. The ether was then distilled on the water bath from the combined filtered ether extracts and the dark brown residue containing much acetic acid was added to ten times its volume of 0·27 N-HCl. After standing overnight the dark brown fatty material was filtered off through a large Büchner funnel, and to the filtrate saturated K acetate solution added until congo red paper no longer turned blue. The solution was then repeatedly extracted with ether until the ether extracts showed only weak porphyrin absorption bands with the hand spectroscope. A bulky interfacial precipitate formed and was roughly separated from the aqueous phase, which, after saturation with solid $(NH_4)_2SO_4$, was exhaustively extracted with repeated small volumes of $CHCl_3$ until the $CHCl_3$ extracts were only pale yellow in colour. The $CHCl_3$ extracts were worked up for stercobilin (Gray, Neuberger & Sneath, 1950). An interfacial precipitate again formed and, together with that formed at the ether-water interface, dissolved in 2·7 N-HCl, giving a dark violet-red solution. The porphyrin in the combined ether extracts was transferred to 2·7 N-HCl and united with the acid solutions of the two interfacial precipitates. The resulting solution was neutralized to pH 3·2 with concentrated NaOH solution and K acetate, and on standing the coproporphyrin separated practically quantitatively; after washing with 2% (v/v) acetic acid it was esterified in the usual way. The final coproporphyrin I tetramethyl ester was recrystallized six times from hot $CHCl_3$-methanol and gave typical crystals m.p. (corr.) 257–259°.

Isolation of hippuric acid. The 4 hr. urine samples were concentrated *in vacuo* to about a quarter of their original volume, acidified to pH 1·0 with approx. 10 N-HCl and extracted with a small amount of $CHCl_3$ which was discarded. The solution was then extracted three times with 2 vol. of ethyl acetate. The ethyl acetate extracts were dried and concentrated to dryness *in vacuo*. The residue, after treatment with charcoal, was recrystallized repeatedly from water until the m.p. (uncorr.) was 190°.

Isolation of haemin. The blood samples were centrifuged and the red cells washed three times with 0·9% (w/v) NaCl. The cells were lysed with distilled water to which a little

saponin was added. In the earlier experiments, crystalline haemin was obtained in the usual way by treatment of the haemoglobin solution with hot glacial acetic acid containing NaCl. Most of the haemin samples were recrystallized from pyridine-$CHCl_3$-acetic acid. In later experiments protoporphyrin methyl ester was prepared by the method of Grinstein (1947) and recrystallized from $CHCl_3$-methanol.

Isotope analyses. The samples, containing at least 0·5 mg. N, were digested by heating for 24 hr. with 1·5 ml. conc. H_2SO_4 containing SeO_2, $HgSO_4$ and K_2SO_4. They were then distilled in the apparatus described by Sprinson & Rittenberg (1949) and the NH_3 converted to N_2. The excess of ^{15}N was determined in the mass spectrometer.

RESULTS

^{15}N *Content of coproporphyrins*

The average excretion of porphyrins in the urine was approximately 50 mg./day and the amount of coproporphyrin eliminated in the faeces was also about 50 mg./day. There was no apparent change following the administration of glycine.

Faecal coproporphyrin. The isotope content of the faecal coproporphyrin (Table 1) rose from 0·243 atom % excess ^{15}N on the 2nd day of glycine feeding to the maximum value of 2·92 atom % which was reached on the 5th day of the experiment, i.e. the 1st day after the feeding of the labelled glycine had been completed. It may be assumed that the ingested glycine mixes very quickly with the glycine 'pool' of the body, and the latter will have the highest ^{15}N content at the end of the 4-day feeding period. The two facts, that the faecal coproporphyrin collected during the 2nd day already contained a large amount of ^{15}N, and that the maximum isotope content was found on the 5th day, would indicate that the time required for the incorporation of the labelled glycine into coproporphyrin and the elimination of this porphyrin into the gut is only between 1 and 3 days.

Table 1. *Isotope contents of faecal coproporphyrin following the administration of isotopic glycine*

(Glycine (12 g.) containing 31·65 atom % excess ^{15}N was fed on days 1–4.)

Days of experiment	^{15}N content (atom % excess)
1	0·07
2	0·243
3	1·56
4	2·41
5	2·92
6	2·51
7	2·21
8	1·42
10	1·10
11	0·92
12	0·78
14–16	0·451
17–20	0·243
21–24	0·177
25–28	0·128
35–39	0·089
75–78	0·031
89–93	0·026
129–135	0·020

The isotope content in the faecal coproporphyrin fell steadily after the 5th day, but even after 130 days there was still a small amount of ^{15}N present in this fraction (Table 1). The values for the period of 5–30 days can be represented reasonably well (Fig. 1) by an equation of the type

$$c_t = 3{\cdot}25e^{-0{\cdot}20(t-t_0)}, \qquad (1)$$

where c_t is the isotope content at time t (in days after the beginning of the experiment); t_0 is the time at which the isotope concentration in the newly formed coproporphyrin is maximal, i.e. some time during the 5th day of the experiment. The values obtained for coproporphyrin after 30 days do not fit equation (1).

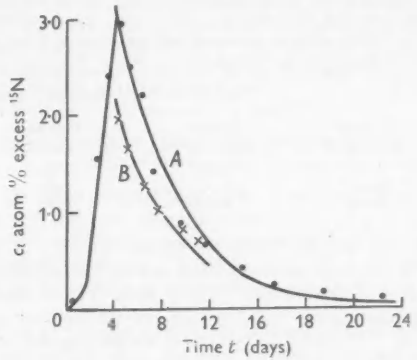

Fig. 1. Comparison of calculated and experimental values for the ^{15}N contents of urinary uroporphyrin and faecal coproporphyrin during the days 4–12 and days 4–24, respectively, after administration of isotopic glycine to a porphyric subject. ×, represents experimental values for uroporphyrin; ●, experimental values for coproporphyrin. Curve A represents the theoretical equation for coproporphyrin: $c_t = 3{\cdot}25e^{-0{\cdot}20(t-t_0)}$; curve B represents the theoretical equation for uroporphyrin:

$$c_t = 2{\cdot}20e^{-0{\cdot}18(t-t_0)};$$

t_0 is the 4th day of the experiment.

Urinary coproporphyrin. Table 2 gives the isotope contents of the urinary coproporphyrin of the first 12 days of the experiment in three 4-day periods and for comparison the corresponding values for faecal coproporphyrin (calculated from the daily figures given in Table 1). The two sets of values for the corresponding periods are of the same order of magnitude, but the figure of ^{15}N for the urinary coproporphyrin during the first 4-day period is higher than for the second 4-day period. This is not

the case with the faecal coproporphyrin. It is probable that this difference is caused by a faster elimination of the porphyrin through the urinary tract than through the alimentary tract.

Table 2. *Comparison of the isotope contents of urinary and faecal coproporphyrin collected during the first* 12 *days of the experiment*

(Glycine (12 g.) containing 31·65 atom % excess ^{15}N were given over the first 4 days. The faecal coproporphyrin values (4-day periods) are mean values calculated from the daily values given in Table 1.)

Days of experiment	^{15}N content (atom % excess) in Urinary coproporphyrin	Faecal coproporphyrin
1–4	2·23	1·07
5–8	1·10	2·50
9–12	0·499	0·90

^{15}N *Content of uroporphyrin*

The isotope content of the urinary uroporphyrin (Table 3), rises more quickly than that of faecal coproporphyrin, the ^{15}N contents on the 2nd day being 1·00 and 0·243 atom % excess respectively.

Table 3. *Isotope contents of urinary uroporphyrin following the administration of isotopic glycine*

(Glycine (12 g.) containing 31·65 atom % excess ^{15}N was fed on days 1–4.)

Days of experiment	^{15}N content (atom % excess)
1	0·370
2	1·00
3	1·50
4	1·90
5	1·95
7	1·26
8	1·08
9–10	0·93
11–12	0·775
75–76	0·086
83	0·054
121–124	0·024
135–137	0·029

This again is most probably explained by the fact that elimination through the kidney is faster than through the gut. However the maximum value for uroporphyrin is definitely lower than that found with coproporphyrin. The values for the days 5–12 can tolerably well be expressed by an exponential equation (Fig. 1).

$$c_t = 2{\cdot}20e^{-0{\cdot}18\,t-t_0}. \qquad (2)$$

As with faecal coproporphyrin, the values for the isotope contents of uroporphyrin collected between 75 and 130 days do not fit the exponential equation (2). In fact, the ^{15}N figures for uroporphyrin found for the period 75–85 days are much higher than those found for faecal coproporphyrin collected during the same period.

Incorporation of ^{15}N *into the haem of circulating haemoglobin in congenital porphyria*

The isotope contents of haemin samples obtained in the first experiment indicated that the patient produced at that time a certain proportion of red cells of an abnormally short life span. Most of the cells, however, had a life span of 100–150 days. These haemin results, together with haematological findings indicating abnormal haemolysis, will be published in a subsequent paper (Gray, Muir & Neuberger, 1950). When a second dose of isotopic glycine was given 7 months later, there was now no haematological evidence for a haemolytic anaemia, and the curve, which was obtained by plotting the ^{15}N contents of haemin samples against time (Fig. 2),

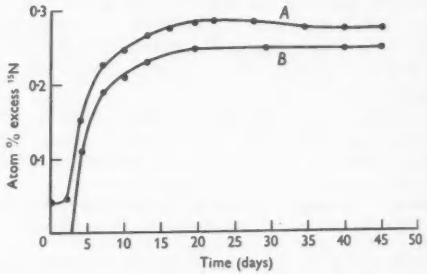

Fig. 2. Experimental and corrected values for the ^{15}N content of the circulating haemin of the porphyric subject at various times after feeding isotopic glycine. Glycine (12 g.) containing 31·65 atom % excess ^{15}N was fed during the first 27·5 hr. of the experiment. Curve *A*, experimental values; curve *B* is corrected for the slow decay of the isotope content of the haemin already present before the second dose of labelled glycine was given. For details of the correction see text.

resembles more closely in shape those reported by Shemin & Rittenberg (1946), by London, Shemin, West & Rittenberg (1949) and by ourselves (see p. 85) for normal man. The protoporphyrin isolated from the blood immediately before the second administration of labelled glycine still contained 0·044 atom % excess ^{15}N (mean of three estimations; individual values were: 0·040, 0·048, 0·044). The uncorrected haemin curve represents therefore both the formation of new cells with high ^{15}N content and the slow destruction of very old cells still labelled from the first experiment. We have assumed, on the basis of the data of Shemin & Rittenberg (1946) and those of London *et al.* (1949), that the fall of ^{15}N in the circulating haem after the 200th day is approximately linear and would amount to 0·0045 atom % excess ^{15}N/day under the conditions of our experiment. We have therefore assumed that the fraction of the ^{15}N of the total circulating haem which is due to old cells decreased linearly from 0·044 to 0·026

atom % excess in the 40 days immediately following the second administration of labelled glycine. The data thus corrected are shown in Fig. 2. The error which might arise from the rather arbitrary character of the correction is not serious for the greater part of the curve.

As shown by Shemin & Rittenberg (1946), the initial part of a normal haem curve fits fairly well an equation of the type

$$c_t = c_0 (1 - e^{-\lambda t}), \quad (3)$$

where λ is a constant.

The corrected data of Fig. 2 are fairly well represented by this equation if the values $c_0 = 0{\cdot}250$ and $\lambda = 0{\cdot}25$ are used.

Incorporation of ^{15}N into the haem of circulating haemoglobin in normal man

Fig. 3 shows the haem curve obtained on feeding an identical amount of ^{15}N in the form of glycine to a normal male subject of body weight similar to that of our porphyric patient. The curve resembles those given for normal man by Shemin & Rittenberg (1946) and London *et al.* (1949). The American workers and ourselves used glycine of very similar isotope content, but the dose given to our two subjects was only about a quarter of that used by the Columbia workers. It is reasonable, therefore, that the maximum ^{15}N content observed by Shemin & Rittenberg (1946) and by London *et al.* (1949) was between 4 and 5 times as great as the corresponding figure in our normal subject.

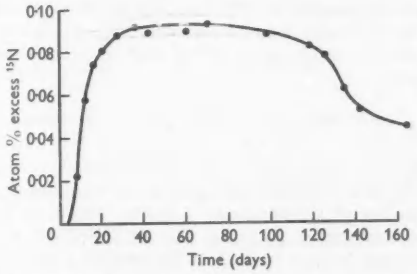

Fig. 3. ^{15}N contents of haemin samples obtained from a normal subject who had been given isotopic glycine over days 1–4. Glycine (12 g.) containing 31·65 atom % excess ^{15}N was fed.

The absolute values of our haemin ^{15}N contents are rather small, and the relative accuracy of the individual analyses is therefore not as high as that attained by the American workers. However, by using the treatment of Shemin & Rittenberg (1946) it can be deduced that the average life span of the red cells in our normal subject is about 130 days. The ascending part of the curve seems to fit an equation

$$c_t = 0{\cdot}090 (1 - e^{-0{\cdot}15t}), \quad (4)$$

from which, as shown by Shemin & Rittenberg (1946), the ^{15}N content, $f(t)$, of the haem formed at time t can be calculated by equation (5)

$$f(t) = \frac{\bar{T}}{100} c_0 \lambda e^{-\lambda t}, \quad (5)$$

where $\bar{T}$ is the average life span.

Using the very approximate figures given above, it can be calculated that the ^{15}N content of the haem formed in the 4–5 days immediately following the administration of the glycine to our normal subject was initially of the order of 1·0–1·2 atom % excess ^{15}N falling to about 0·6–0·7 atom % excess ^{15}N at the end of that period.

Incorporation of ^{15}N into hippuric acid in the normal and porphyric subject

The urine obtained in the two experiments carried out with the normal subject was collected over short periods, and the hippuric acid samples isolated and analysed separately. As shown in Table 4, the bulk of the hippuric acid is excreted over the first 4 hr.

Table 4. *Isotope contents of hippuric acid samples obtained from a normal subject following the administration of isotopic glycine*

(Glycine (12 g.) containing 31·65 atom % excess ^{15}N was fed over days 1–4. In the first benzoate experiment, the subject took by mouth 3 g. Na benzoate at 8 a.m. on the 5th day after the beginning of glycine feeding. In the second benzoate experiment, the subject took by mouth the same dose of benzoate at 11.15 a.m. on the 7th day after the beginning of glycine feeding.)

Periods of urine collection after benzoate administration (hr.)	Yield of hippuric acid (g.)	^{15}N Content of hippuric acid (atom % excess)
	Experiment 1	
0–4	2·213	0·880
4–8	0·180	0·650
8–12	0·080	0·544
12–16	0·030	0·561
16–24	0·213	0·470
	Experiment 2	
0–4	2·07	0·306
4–8	0·076	0·257
8–12	0·015	0·250
12–20	0·014	0·353

The isotope content in the first benzoate feeding falls steadily with time, whilst in the second benzoate experiment this effect is not marked. A direct comparison of the isotope contents of newly formed porphyrin and of hippuric acid is not possible in the normal, but the values calculated from the initial part of the haem curve (Fig. 3) indicate that the protoporphyrin formed on the 5th and the 7th day had about 1·0–1·2 and 0·65–0·85 atom % excess

respectively. These values are about 1·5–2·5 times greater than the ^{15}N contents of the hippuric acid excreted on these days.

In the porphyric the excreted porphyrins may be expected, as discussed above, to give a direct but approximate measure of the isotope contents of newly formed porphyrins. A comparison between ^{15}N contents of coproporphyrins and of hippuric acid is shown in Fig. 4. The values for coproporphyrin

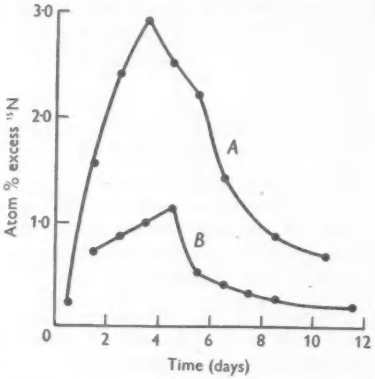

Fig. 4. Comparison of ^{15}N contents of hippuric acid (*B*) and of faecal coproporphyrin (*A*) after feeding glycine (12 g.) containing 31·65 atom % excess to a porphyric over days 1–4.

have been plotted on the assumption that the interval between the formation of coproporphyrin and its elimination in the faeces is 1 day. The conclusion would not be greatly affected, if the delay is somewhat longer, i.e. 2–3 days. It is clear from Fig. 4 that the isotope contents of the hippuric acid samples are much smaller than those of coproporphyrin produced at similar times of the experiment.

DISCUSSION

Comparison of the isotope contents of the different porphyrins of the porphyric

When isotopically labelled glycine is administered it will mix with the free glycine present in the tissues, be synthesized into proteins, partake in various reversible reactions such as synthesis of glutathione and serine and also take part in irreversible reactions such as the formation of uric acid and porphyrins. In biological isotope experiments, one or more of such metabolic products of glycine are isolated at various times after the administration of the labelled compound, and the isotope contents estimated. For the interpretation of the results of such experiments it is convenient to use the term glycine 'pool'. This glycine 'pool' will consist not only of free glycine present as such in the organism, but will also include a proportion of glycine contained in proteins and a certain fraction of the other compounds which can be converted into glycine. In fact, the glycine 'pool' cannot at present be identified with any definite fraction of compounds present in tissues.

If all four nitrogen atoms of protoporphyrin IX, of coproporphyrin I and uroporphyrin I are derived exclusively from glycine, and if all these porphyrins are formed in the bone marrow, then the isotope contents of these porphyrins formed at the same time should be identical and reflect accurately the isotope content of the glycine present in the marrow at that time. The ^{15}N content of the excreted coproporphyrin and uroporphyrin is not necessarily an exact measure of the isotope content of the newly formed porphyrin. If the two porphyrins are stored at all in the body then the labelled newly formed material will be diluted to some extent by preformed porphyrin. Evidence, in fact, exists for such a storage (Fischer, Hilmer, Lindner & Pützer, 1925) and the experimental data on the excreted porphyrins therefore represent minimal figures for the ^{15}N contents of the newly formed porphyrins. Such a storage would also explain the high ^{15}N contents found particularly for uroporphyrin between the 70th and 135th day of the experiment, when the label must have almost completely disappeared from the glycine 'pool' of the body. The difference in isotope contents of the two porphyrins indicates that storage of uroporphyrin is more extensive than that of coproporphyrin.

A comparison of ^{15}N contents of the excreted porphyrins with those of the newly formed porphyrin of the circulating haem will be made in a subsequent paper.

Comparison of the glycine 'pool' of the normal with that of the porphyric

In the normal subject the highest isotope content of the newly formed protoporphyrin was calculated to be about 1·0–1·2 atom % excess. The porphyric who received an identical amount of ^{15}N showed a maximal isotope content in his various porphyrins of about 3·0–3·5 atom % excess. This estimate is given either by the coproporphyrin results directly or by the haemin values. The difference between the isotope content of the haem in the porphyric and in the normal is also apparent from a comparison of Figs. 2 and 3. It would appear that the ingested labelled glycine is diluted to a greater extent in the normal than in the porphyric, so that the glycine 'pool' of the latter appears to be only about half that of the normal subject investigated. It may also be significant that the rate of decrease of the isotope content of the newly formed haem and of the other porphyrins (which is given by the constant λ of the exponential equations (2)–(4)) is greater than those

found in normals by Shemin & Rittenberg (1946) and London *et al.* (1949). This would indicate that the turnover rate of the glycine 'pool' is greater in the porphyric than has been found so far in normal subjects.

Comparison between the isotope contents of hippuric acid and those of porphyrins

In man, hippuric acid is formed mainly in the liver. This organ contains appreciable amounts of free glycine and other substances like glutathione which can be rapidly converted into glycine. It was expected that the glycine in the liver, which is probably the main precursor of hippuric acid, would have the same isotope content as the glycine in bone marrow which is utilized for the synthesis of porphyrins. However, the present results show clearly that this assumption is not correct. It is likely that benzoic acid mobilizes glycine of low isotope content or induces synthesis of glycine from nitrogen-free precursors. This would also explain the decrease of ^{15}N in successive fractions of hippuric acid isolated in the first experiment with the normal subject. Benzoic acid will probably first combine with free glycine in the liver and reserves will only be mobilized when the liver glycine is largely used up. The ^{15}N content of hippuric acid cannot, at least with the dose used in the present experiments, be employed for estimating the isotope content of the glycine 'pool' of man.

SUMMARY

1. Glycine containing 31·65 atom % excess ^{15}N was fed to a patient suffering from congenital porphyria. The faecal and urinary coproporphyrin samples isolated during the first few weeks of the experiments had high ^{15}N contents which decreased with time. The urinary uroporphyrin samples had somewhat lower ^{15}N contents.

2. It is suggested that the differences in isotope content observed may be explained by differences in the degree of storage of uroporphyrin and coproporphyrin in the body.

3. Sodium benzoate was administered to a normal man and to the porphyric. The ^{15}N contents of the hippuric acid isolated indicate that administration of benzoic acid stimulates a mobilization of glycine from proteins, or the synthesis of glycine from nitrogen-free precursors, or both.

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Studies in Congenital Porphyria

2. INCORPORATION OF ^{15}N IN THE STERCOBILIN IN THE NORMAL AND IN THE PORPHYRIC

By C. H. GRAY, A. NEUBERGER and P. H. A. SNEATH
Department of Chemical Pathology, King's College Hospital, Denmark Hill, London, S.E. 5, and the National Institute for Medical Research, Hampstead, London, N.W. 3

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In the course of the experiments described in the preceding paper (Gray & Neuberger, 1950), samples of stercobilin were isolated from the faeces of the porphyric. The surprising observation was made that the isotope content of the stercobilin was extremely high during the first few days of glycine feeding. This observation, together with similar observations in the normal, are the subject of the present paper. Some of the results have already been reported (Gray, Neuberger & Sneath, 1949).

EXPERIMENTAL

Details of the two experimental subjects have been given in the first paper of this series (Gray & Neuberger, 1950).

Isolation of stercobilin hydrochloride from faeces of the porphyric. The $CHCl_3$ extract from the preparation of the

faecal coproporphyrin (Gray & Neuberger, 1950) was extracted with water (30 × 1/50 vol.). The combined aqueous extracts were rendered 3% (w/v) in respect of HCl by the addition of the required volume of 36% (w/v) HCl. The stercobilin hydrochloride was extracted with $CHCl_3$, which was then filtered through a $CHCl_3$-moistened paper and evaporated to dryness on the water bath. On the addition of a small volume of acetone, the stercobilin hydrochloride precipitated out and was centrifuged from the mother liquor containing brown impurities. After six recrystallizations from $CHCl_3$, crystalline stercobilin hydrochloride was obtained, the yield being higher in the porphyric than in the normal. This method is a modification of that of Watson (1934).

Isolation of stercobilin hydrochloride from faeces of a normal subject. The faeces were worked up in 4- and 6-day batches in a similar manner to that employed with the faeces of the porphyric. However, as the amount of porphyrins present was so much smaller, only four ether extractions were necessary before $CHCl_3$ extraction of the stercobilin. $(NH_4)_2SO_4$ was not added to assist $CHCl_3$ extraction of the stercobilin, since tarry impurities were more readily extracted when $(NH_4)_2SO_4$ was present. Before recrystallization, the stercobilin hydrochloride was precipitated from $CHCl_3$ solution with light petroleum.

RESULTS

The incorporation of ^{15}N *into faecal stercobilin of normal man*

The stercobilin curve of the normal subject (Fig. 1) shows two distinct peaks, one occurring immediately after the glycine feeding and the other with its maximum at or near the 138th day. Between these two peaks, the curve is quite flat, but the stercobilin excreted during the whole of this period contained significant amounts of ^{15}N.

In order to interpret the results obtained with stercobilin it is necessary to follow the simultaneous changes in the ^{15}N content of the haem of the circulating haemoglobin. The haemin curve of our normal subject (Gray & Neuberger, 1950, Fig. 3) shows that on the 5th day of the experiment, i.e. on the 1st day after glycine feeding had been completed, there was no detectable excess of ^{15}N in the haem. On the 8th day of the experiment the haem contained 0·022 atom % excess ^{15}N. This means that there is an interval of about 3 days between the peak of the isotope content of the glycine pool (which occurs presumably at the end of the 4th day) and the appearance of a significant amount of ^{15}N in the circulating haem. This delay is probably associated with the production of protoporphyrin in the primitive red cell and the maturation of this cell in the bone marrow. In contrast, the stercobilin excreted during the first 4-day period, during which isotopic glycine was being fed, had 0·085 atom % excess ^{15}N. The sample collected during the second 4-day period during which the ^{15}N in the haem began to rise, had 0·161 atom % excess ^{15}N. During the next 12 days the stercobilin fell while the haem curve rose steeply. The second flat part of the stercobilin curve corresponded to the flat part of the haemin curve (30–90 days) and apparently during this period no breakdown of haemoglobin occurred. However, stercobilin samples collected during this period contained an average of 0·020 atom % excess ^{15}N.

During the third period (90–160 days) circulating haemoglobin was destroyed as shown by the decrease of ^{15}N. The highest rate of breakdown was between the 120th and 150th day of the experiment. Our values for ^{15}N in the haem are too small to allow accurate plotting of the function dc/dt, the rate of change of the isotope content of the haem against time t. But it appears that in our normal subject such a function would be represented by a curve with a maximum at or near $t = 135$ days, and it is likely from the results of Shemin & Rittenberg (1946) that such a curve would be symmetrical.

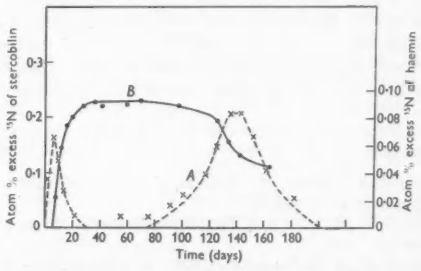

Fig. 1. ^{15}N contents of stercobilin hydrochloride samples (curve *A*) obtained at various times after the administration to a normal subject of glycine (12 g.) containing 31·65 atom % excess ^{15}N. Experimental points (×) represent mean values of 4- or 6-day collections. Glycine was given over days 1–4. The broken line for the values of 80–180 days is a calculated normal distribution curve with a mean value of 0·210 at the 140th day and a standard deviation of 18 days. The ^{15}N contents of the haemin samples (full line; experimental values ●) of the same subject (curve *B*) are given for comparison. The values are taken from the earlier paper (Gray & Neuberger, 1950, Fig. 3).

After the 90th day the ^{15}N content of the stercobilin rose steadily to reach a maximum between the 135th and 140th day. This was almost concomitant with the greatest rate of fall in the ^{15}N content of the circulating haem. The correlation between the two sets of values is so good that there can be no doubt that most or all of the labelled stercobilin excreted between the 100th and 160th day was derived from the breakdown of circulating haemoglobin. No data are available beyond the 180th day, but it appears that the values of Fig. 1 lying between 110 and 160 days can be represented

by a normal distribution curve with a standard deviation of 18. This of course must be an approximation, even if the frequency of deaths amongst red cells were to follow exactly a normal distribution curve. Since the isotopically labelled cells were not all formed at the same instant, a distribution derived directly from the data of Fig. 1 cannot be exactly normal. However, if the approximation is accepted, it would follow that about 68 % of all cells die between 112 and 142 days after their appearance in the circulation. London, Shemin, West & Rittenberg (1949) calculated from the rate of decrease of isotope in the haemin in two different subjects that about 50 % of the cells died between the 106th and 141st day and 91st and 123rd day respectively.

The incorporation of ^{15}N into faecal stercobilin of porphyric

The results obtained with the porphyric are shown in Fig. 2. Comparison with the results obtained in the normal (Fig. 1) show that the first

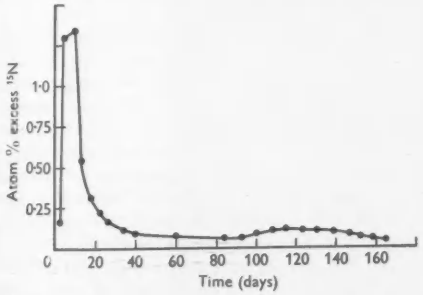

Fig. 2. ^{15}N contents of stercobilin hydrochloride samples obtained at various times after administration to a congenital porphyric of glycine (12 g.) containing 31·65 atom % excess ^{15}N. Experimental points represent 4-day collections. Glycine was fed over days 1–4.

peak is greatly raised, while the second peak is barely noticeable. Comparison of the isotope contents of the stercobilin with those of faecal coproporphyrin for the same periods (Gray & Neuberger, 1950) shows that for the first 8 days the coproporphyrin figures are somewhat higher than those of stercobilin, but subsequently the reverse is the case. In the preceding paper, evidence was presented to show that the isotope contents of the excreted porphyrins are only slightly lower than those of the newly formed haem. The high ^{15}N content of the stercobilin during the initial period would therefore indicate that, in contrast to the normal, almost all of the stercobilin excreted by the porphyric must be derived from some source other than the red cells of normal life span. This is also shown by the fact that at the period 110–150 days when the ^{15}N content of the circulating haem decreases rapidly, the isotope content of the stercobilin rises only very slightly. The possibility that this high ^{15}N content in the initial part of the experiment may be caused by destruction of red cells of abnormally short life span was considered especially in view of the fact that evidence was obtained that a haemolytic episode was taking place during the first glycine experiment. However, in the second experiment where no evidence of a gross haemolytic process was present and the haem curve was apparently normal, the isotope content of the stercobilin collected between the 3rd and 8th day of the experiment was 1·24 atom % excess, almost identical with that found in the first experiment. It therefore appears that the high ^{15}N content of the stercobilin cannot be due to any large extent to the breakdown of red cells of extremely short life span.

Another less striking difference between the normal curve and that of the porphyric is the relatively high ^{15}N content of the stercobilin sample collected between the 30th and 90th day. This may possibly be related to the smaller glycine 'pool' in the porphyric (see Gray & Neuberger, 1950).

It is of interest to note that the initial part of the stercobilin curve after the 4th day of the experiment cannot be fitted to an exponential equation. The curve approximates more closely to a hyperbola, but the physical significance of this is obscure.

Comparison of stercobilin obtained from porphyric with that obtained from non-porphyric patients

The unexpected results described above of the high ^{15}N content of the stercobilin of the porphyric subject during the first days of the experiment suggested the possibility that the stercobilin excreted by the porphyric might be derived from the type I porphyrin or might contain more than two carboxyl groups. A comparison was therefore carried out between the stercobilin excreted by the porphyric and samples obtained either from a normal subject or from patients suffering from haemolytic anaemia (the latter presumably excrete stercobilin of normal structure).

Melting points. Stercobilin samples were dried first over paraffin wax and then over P_2O_5 *in vacuo* at 65°. All samples of stercobilin hydrochloride started sintering at about 120° and melting appeared to be complete at 140°. This agrees with the observations of Watson (1934).

Analyses. Elementary analyses of stercobilin hydrochloride, as shown by Fischer & Halbach (1936), give results too low for C and H, but too high for Cl. This was explained as being due to the retention by the crystals of small amounts of $CHCl_3$ which could not be removed by prolonged drying. Similar results were obtained in the present work; no differences were found between 'normal' and 'porphyric' stercobilin. (Found for sample 1 ('normal'): C, 59·9; H, 7·1; N, 9·0; Cl, 7·0. Found for sample 2 ('porphyric'): C, 60·0; H, 7·2; N, 8·7; Cl, 6·8. Calc. for $C_{33}H_{46}O_6N_4HCl$: C, 62·8;

H, 7·5; N, 8·9; Cl, 5·6 %.) These analytical figures are almost identical with those obtained by Fischer & Halbach (1936).

Specific rotations. Sample 1 ('normal') had $[\alpha]_D^{27°} -3770 \pm 70°$ in $CHCl_3$ (c, 0·1); sample 2 ('porphyric') had $[\alpha]_D^{27°} -3670 \pm 70°$ in $CHCl_3$ (c, 0·09).

Visible and ultraviolet absorption. In 0·1 N-HCl both samples possessed identical absorption curves showing maxima at 490 mμ. ($\epsilon = 9{\cdot}3 \times 10^4$) and at 364 mμ. ($\epsilon = 0{\cdot}79 \times 10^4$). There was no significant difference in the spectral absorption curve in the visible or near ultraviolet regions.

Infrared absorption. Two specimens of stercobilin, one presumed to be normal and the other obtained from the porphyric, were examined by Dr N. Sheppard of the Department of Colloid Science, University of Cambridge. Spectra

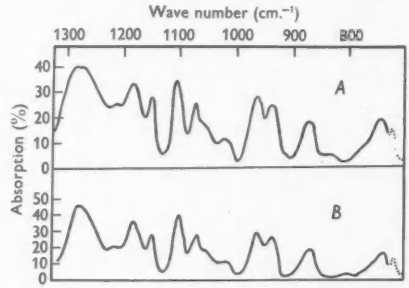

Fig. 3. Comparison of the infrared spectrum of stercobilin hydrochloride obtained from a normal subject (curve *A*) with that obtained from a porphyric subject (curve *B*). The samples were in Nujol suspension; the normal stercobilin was a thick suspension whilst the other was a medium suspension.

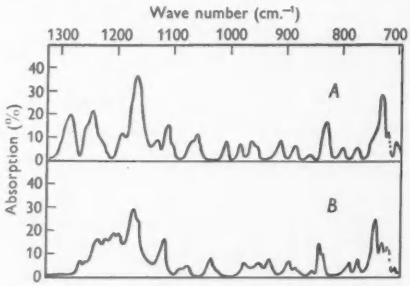

Fig. 4. Comparison of the infrared spectra of coproporphyrin I methyl ester (curve *A*) and of coproporphyrin III methyl ester (curve *B*). The samples were in Nujol suspension. (The spectra of the two esters were identical between 3500 and 1300 cm.$^{-1}$, with the exception that the coproporphyrin III ester had an extra weak band at about 1350 cm.$^{-1}$.)

were taken between 3000 and 700 cm.$^{-1}$, only the region between 700 and 1300 cm.$^{-1}$ being shown on Fig. 3. No differences between the two specimens were observed in the region above 1300 cm.$^{-1}$, but there were very slight differences near 830 and 800 cm.$^{-1}$. In Dr Sheppard's opinion, these very slight differences might be related to 'slight overall changes in molecular structure' or be caused by the presence of small amounts of impurities. The latter explanation appears more probable since the spectra of coproporphyrin methyl esters of series I and III which differ in structure in the same way as normal and the possible 'abnormal' stercobilin differed considerably in the region 1500–700 cm.$^{-1}$ (Fig. 4). The weight of evidence obtained from the infrared spectra therefore favours the assumption that these two stercobilin samples have identical structures. The elementary analysis proves that the stercobilin obtained from the porphyric does not contain more than two carboxyl groups.

DISCUSSION

The origin of stercobilin in the normal subject

Evidence for three metabolic fractions of stercobilin. The results obtained with ^{15}N suggest that stercobilin consists of three separate fractions of different metabolic origin.

The third and largest fraction is derived from the breakdown of red cells of normal life span and is represented in Fig. 1 by the peak which has a maximum at or near the 138th day.

The first peak has a maximum during the second 4-day period, when the isotope content of the glycine 'pool' of the body is also highest. The slope of that part of the curve (Fig. 1) which extends from the time of the second faeces collection (5th–8th day of the experiment) to the 20th day corresponds in shape very closely to a curve calculated for the decrease of ^{15}N in the glycine 'pool' of the body. The latter is obtained from the haem results on the same subject (Gray & Neuberger, 1950) assuming that the ^{15}N content of newly formed circulating haem reflects approximately the isotope content of the glycine 'pool'. This correspondence between the two curves and the relatively high isotope content of the stercobilin collected during the first 4 days of the experiment, when the circulating haem is devoid of isotope, proves that this first stercobilin fraction cannot be derived from circulating haemoglobin, but must originate from some other precursor by a very fast reaction. The time taken for the transformation of the nitrogen of glycine to that of this first stercobilin fraction cannot be accurately assessed, but must be less than 4 days. The excretion of bile, the transformation of bilirubin to stercobilin and the elimination of the latter in the faeces may occupy 1–2 days.

London, West, Shemin & Rittenberg (1948) have also briefly reported the incorporation of ^{15}N in the stercobilin shortly after administration of isotopic glycine to a normal subject.

The continuous excretion of stercobilin with a small, but apparently constant, ^{15}N content between 20 and 80 days suggests that there is a second stercobilin fraction, the metabolic origin of which may differ from that of the first and the third fractions. The haem results suggest that no normal red cells

are destroyed to any significant extent until they reach the age of about 80 days. This second fraction cannot therefore be derived from circulating haemoglobin. It is also unlikely that the first and second fractions are formed by the same mechanism. The isotope content of stercobilin collected near the 60th day is 0·021 atom % excess as compared with a value of 0·161 atom % excess for the second 4-day period. The assumption that the two fractions are formed by the same reaction would imply that the isotope content of the glycine 'pool' had only decreased to about one-eighth in this intervening period. This is obviously not the case. Some bile pigment is stored in the liver and in the gall bladder, and this may lead to a reduction of isotope content of the stercobilin in the beginning of the experiment and an increase in the later stages. However, on this basis we should expect a tailing off of the ^{15}N content of the stercobilin between the 20th and 80th day. This is not the case, and it seems, therefore, probable that the second stercobilin fraction is formed by a separate mechanism.

The relative proportions of the three fractions. The relative amounts of the three stercobilin fractions can be assessed by a graphical integration of the areas enclosed by the extrapolated curves (Fig. 1, broken lines). Such an estimate can only be approximate, since the degree of overlapping cannot be ascertained. However, it appears that about 70 % of the excreted stercobilin originates from the breakdown of circulating haemoglobin (fraction 3), 15–20 % is formed by a fast reaction (fraction 1) and the residual 10–15 % by a much slower reaction (fraction 2). This estimate is supported by other data. At a time when the breakdown of haemoglobin is at its maximum, the rate of decrease of ^{15}N in the total circulating haem is about 0·002 atom % excess/day. Since a fraction corresponding to $1/\overline{T}$ (where $\overline{T}$ is the average life span) is broken down each day, the ^{15}N content of the haem which is converted to bile pigment during that period should have an isotope content of $0{\cdot}002 \times 130$ which is 0·260 atom % excess. The experimental value for the stercobilin excreted at this time was 0·210 atom % excess, suggesting that at least 20 % of the stercobilin was derived from a precursor which contains little or no label. The average isotope content of porphyrins formed during the second 4-day period has been assumed on the basis of the haem results to be about 0·8 atom % excess. The stercobilin excreted during that time had 0·16 atom % excess ^{15}N. It would thus appear that the stercobilin which forms fraction 1 comprises about 20 % of the total.

Metabolic sources of the stercobilin. There are three possible sources of the two fractions of the stercobilin which give rise in the isotope experiments to a relatively high ^{15}N content in the beginning and to a fairly constant, but low, ^{15}N content in the middle of the experiment. It is possible that porphyrins or haems are not involved as intermediates and the bile pigment may be formed directly by condensation of pyrrolic or dipyrryl compounds. This is unlikely, since the arrangement of the side chains in bilirubin and stercobilin resembles completely that found in protoporphyrin IX. There is no reason to expect the same arrangement of side chains in stercobilin, if it were formed by direct condensation of monocyclic or dicyclic compounds. If, however, a linear tetrapyrrolic compound were an intermediate in porphyrin synthesis, such a precursor might be directly converted to biliverdin or bilirubin and thence to stercobilin. The second possibility is that a part of the stercobilin is associated with porphyrin formation outside the erythropoietic system. Cytochromes and catalase are present in almost all animal cells and we may assume that these haem proteins are formed *in situ*. The concentrations of these proteins in the human body are very low compared with that of haemoglobin (Drabkin, 1948), but the turnover rates of such porphyrins may be high. Catalase and cytochrome *c* contain protoporphyrin IX and a modified protoporphyrin respectively, and these proteins may therefore give rise to bilirubin. The oxidation of some of the other cytochromes is likely to produce bilirubin of abnormal structure. Quantitatively the most important haem protein other than haemoglobin is myoglobin, which in man accounts for about 5 % of the total porphyrin (Drabkin, 1948). Its turnover rate in animals, however, appears to be low, as judged from experiments with isotopic iron (Theorell, communication at the First International Congress of Biochemistry, Cambridge 1949). It is suggested, therefore, that the metabolism of porphyrins derived from myoglobin, catalase and possibly cytochrome *c* may account for the ^{15}N content of the stercobilin excreted between the 30th and 80th day, but only for a small proportion of the high isotope content found in the initial period of the experiment.

Finally, it is possible that the first fraction of the stercobilin may in fact be associated with the process of red-cell formation. It has been suggested (Gray *et al.* 1949) that normal red cells have a certain 'infantile mortality', i.e. a certain proportion of cells is normally destroyed before being released into the circulation. There is no evidence, so far as we are aware, for such a hypothesis, but it cannot be excluded. A more probable explanation is as follows: as shown by Thorell (1947) the synthesis of protein in the red cell precedes that of porphyrins. However, there is likely to be a stage during which the red cell behaves like any normal growing cell with respect to porphyrins or haems. During this phase of development both synthesis and destruction of porphyrins will take place, the rate of the latter being smaller than that of the former with the net

result that the total amount of porphyrin increases. The discarded haem or porphyrin will be transformed to bilirubin and stercobilin. On the basis of this hypothesis bile-pigment formation is normally associated with erythropoiesis and an increased excretion of stercobilin may indicate either an increased rate of destruction of red cells or an increased rate of formation. The speed with which the labelled glycine is converted to stercobilin agrees with this explanation.

Stercobilin formation in the porphyric

From the evidence produced it appears almost certain that the stercobilin excreted by the porphyric has a normal structure. The results obtained with ^{15}N indicate that the fraction of the stercobilin which in the normal represents about 70% of the total, and is derived from the breakdown of circulating haem, is only a small fraction (possibly about 10–15%) of the stercobilin excreted by the porphyric. Most of the stercobilin is derived from a precursor which is itself formed from glycine at a very fast rate. It is reasonable to assume that this precursor is identical with that of fraction 1 in the normal and that one of the features of congenital porphyria is a greatly increased rate of formation of this as yet unknown precursor. With respect to the origin of the main fraction of the stercobilin there are the same three possibilities which are discussed above in connexion with the source of fraction 1 of stercobilin in the normal. Haematological findings and other isotope results will be presented later, and the stercobilin figures obtained with the porphyric will be discussed in some detail.

Grinstein, Aldrich, Hawkinson & Watson (1949) in a preliminary note have also reported a high rate of incorporation of ^{15}N into the coproporphyrin, uroporphyrin and stercobilin during the first few days after feeding labelled glycine to a congenital porphyric.

SUMMARY

1. The ^{15}N contents of stercobilin samples collected over a period of 180 days after feeding labelled glycine to a normal subject have been determined. The highest ^{15}N contents were observed in samples obtained between the 120th and 150th day of the experiment, at a time when the rate of breakdown of the labelled circulating haem was highest.

2. A second peak was observed in the first few days of the experiment; this is assumed to be caused by a breakdown of haemoglobin during the maturation of the red cell. Other explanations for this phenomenon are discussed.

3. The stercobilin samples obtained between the 30th and 100th day of the experiment contained small amounts of ^{15}N. It is suggested that this fraction may largely represent bile pigment formed from haem proteins other than haemoglobin.

4. In a similar experiment with a congenital porphyric it was found that the isotope content of the stercobilin was very high in the first 2–3 weeks of the experiment; the peak corresponding to the breakdown of circulating haemoglobin was not very marked.

5. Evidence is presented to indicate that the stercobilin excreted by the porphyric has the same structure as that obtained from normal subjects.

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The Rate of Enzymic Hydrolysis of Phosphoric Esters

3. CARBOXY-SUBSTITUTED PHENYL PHOSPHATES

By P. G. WALKER and E. J. KING
Postgraduate Medical School, London, W. 12

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Delory & King (1943) published a comparative study of the hydrolysis by faecal alkaline phosphatase of a series of phosphoric esters and established a correlation between the pK values of their second phosphate dissociations, the optimum pH for the enzymic hydrolysis and the Michaelis constants of the esters. They showed that the more acid this phosphate dissociation, the more alkaline was the pH optimum, the greater was the amount of hydrolysis and the lower the Michaelis constant. They found their results consistent with the Martland & Robison (1927) theory of enzyme-substrate combination and with Kay's (1932) interpretation of the pH optima of alkaline phosphatase for different substrates as a balance between increase in the rate of hydrolysis and increase in the rate of enzyme inactivation with increasing alkalinity.

Two more esters, the *o*- and *p*-carboxyphenyl phosphates have been added to this series. For their synthesis the carboxyl groups of salicylic and *p*-hydroxybenzoic acid had to be protected by esterification before phosphorylation of the phenolic hydroxyl group by phosphorus oxychloride. Even then the usual conditions employed for this reaction (King & Nicholson, 1939) were too drastic, and the reaction mixture had to be kept at less than 10° throughout the phosphorylation. The crystalline esters so obtained had a much lower barium and higher phosphorus content than was expected; and when potentiometric titration showed only one titrable hydrogen per atom of phosphorus, and that with a pK of about 1, it was realized that the product was a pyrophosphoric ester and not an orthophosphate. Only later was it discovered that the conditions we had been forced to adopt for the phosphorylation were almost exactly those described by Neuberg & Wagner (1926) for the synthesis of diphenyl pyrophosphate.

Mild alkaline hydrolysis of these pyrophosphates split the pyrophosphate link, and set free the carboxyl group. The carboxyphenyl phosphates were isolated as barium salts; and titration now showed the three dissociations expected, which were ascribed in order of decreasing acidity to the primary phosphate, the carboxyl group and the secondary phosphate dissociations (Table 1).

Table 1. pK *values of carboxyphenyl phosphoric esters*

o-Carboxyphenyl phosphate	pK_1, 0·95; pK_2, 3·50; pK_3, 6·11
p-Carboxyphenyl phosphate	pK_1, 1·14; pK_2, 3·90; pK_3, 6·40
Di-*o*-carbomethoxyphenyl pyrophosphate	$pK_{1 \text{ and } 2}$, 1·05
Di-*p*-carbethoxyphenyl pyrophosphate	$pK_{1 \text{ and } 2}$, 1·10

The pH optima for hydrolysis by faecal alkaline phosphatase and the Michaelis constants for these esters were determined, and the results are included in Table 2, which also contains the corresponding data for some of the esters investigated by Delory & King (1943).

EXPERIMENTAL AND RESULTS

Preparation of esters

Pyrophosphoric esters

The procedure of King & Nicholson (1939) was followed, except that the reaction mixture was cooled in an ice bath throughout the addition of $POCl_3$ to the pyridine solution of

Table 2. *Rates of enzymic hydrolysis of phosphoric esters*

Phosphoric ester	Secondary phosphate pK	Optimum pH	Rate of hydrolysis*	Michaelis constant (K_m)
p-Bromophenyl	5·44	9·96	3·4	0·0003
Phenyl	5·73	9·73	2·4	0·0006
o-Carboxyphenyl	6·11	9·10	1·6	0·0010
β-Glycero	6·34	8·82	1·0	0·0012
p-Carboxyphenyl	6·40	8·00	0·4	0·0030
Ethyl	6·45	8·08	0·3	0·0025

* Compared with β-glycerophosphate taken as 1; substrate concentrations 0·01 M.

the carboxyl ester, and instead of refluxing for 10 min. it was allowed to stand 24 hr. in the ice chest, and was again cooled during the stage of precipitation with $Ba(OH)_2$. The Ba salts were recrystallized from hot aqueous ethanol, the *di-o-carbomethoxyphenyl pyrophosphate* forming fine needle-like crystals, and the *di-p-carbethoxyphenyl pyrophosphate* glancing platelets.

Analyses. Di-o-carbomethoxyphenyl pyrophosphate. (Found: C, 32·0; H, 2·9; P, 10·4; Ba, 24·0. $C_{16}H_{14}O_{11}P_2Ba$ requires: C, 33·0; H, 2·4; P, 10·3; Ba, 23·5%.) *Di-p-carbethoxyphenyl pyrophosphate.* (Found: C, 34·8; H, 2·9; P, 10·3; Ba, 22·6. $C_{18}H_{18}O_{11}P_2Ba$ requires: C, 35·4; H, 3·0; P, 10·1; Ba, 22·5%.)

Orthophosphoric esters

After dissolving in water and removal of Ba by the calculated amount of H_2SO_4, hydrolysis of the pyrophosphate esters was accomplished by the addition of five equivalents of NaOH. After 18 hr. at 37° only two equivalents of NaOH remained (titration with HCl to phenolphthalein end point). No inorganic phosphate was liberated by this hydrolysis. The carboxyphenyl phosphates were isolated by the addition of $BaCl_2$, either as the $Ba_{1·5}$ salts from alkaline solution or as the half Ba salts from acid (pH <3) solution.

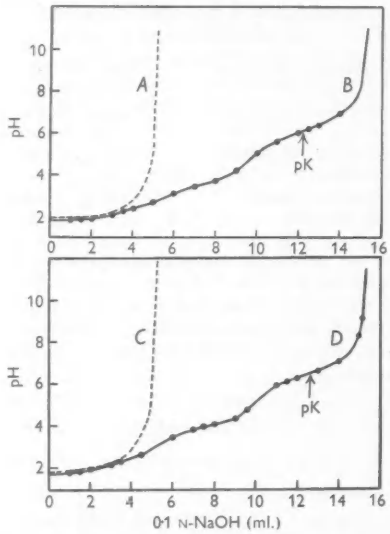

Fig. 1. Dissociation curves for *A*, *o*-carbomethoxyphenyl pyrophosphate; *B*, *o*-carboxyphenyl phosphate (pK 6·11); *C*, *p*-carbethoxyphenyl pyrophosphate; and *D*, *p*-carboxyphenyl phosphate (pK 6·40).

Analyses. For the isomeric $Ba_{1·5}$ salts of the carboxyphenyl phosphates. (Found for *barium o-carboxyphenyl phosphate*: P, 7·7; Ba, 49·0; and for *barium p-carboxyphenyl phosphate*: P, 7·6; Ba, 47·8. $C_7H_4O_6PBa_{1·5}$ requires: P, 7·4; Ba, 48·7%.)

Titration curves. For the pH values a Muirhead pH meter with a dipping glass electrode was used. The meter was set with standard phthalate (pH 4·00) and borate (pH 9·00) buffers. The Ba pyrophosphate ester (0·25 mmol.) or the Ba orthophosphate ester (0·5 mmol.) was dissolved in a carefully measured volume of water, and treated with the exact amount of H_2SO_4 to precipitate the Ba. The solution of the acid was then titrated with CO_2-free 0·100 N-NaOH (temp. 18–20°). Three values for each dissociation constant were calculated using the equation $Ka = \frac{[H^+] \times (B + [H^+])}{C - (B + [H^+])}$ for pK_1 and pK_2, and the equation $pK = pH - \log B/(C - B)$ for pK_3 (when H^+ is negligible compared with B and C (see Van

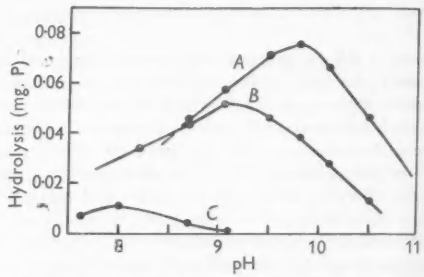

Fig. 2. Optimum pH for hydrolysis by phosphatase of *A*, phenyl phosphate (pH 9·7); *B*, *o*-carboxyphenyl phosphate (pH 9·1); and *C*, *p*-carboxyphenyl phosphate (pH 8·0).

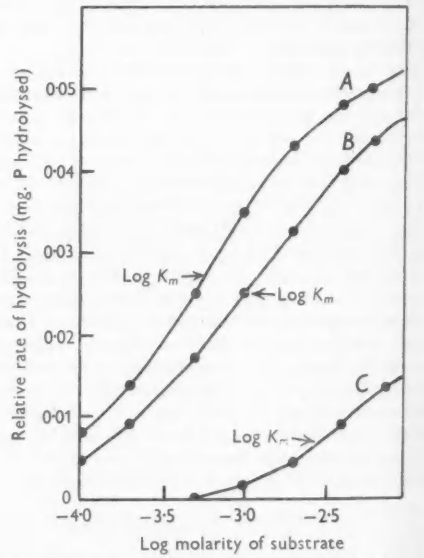

Fig. 3. Curves showing Michaelis effect of phosphoric esters and faecal phosphatase at the optimum pH for each ester. The continuous lines are drawn from the equation $\log\frac{1}{x} = \log\frac{1}{K_m} + \log\left(\frac{V}{v} - 1\right)$, the values of the constant K_m being chosen for the best fit for the determined values. *A*, phenyl phosphate (K_m 0·0006); *B*, *o*-carboxyphenyl phosphate (K_m 0·0010); *C*, *p*-carboxyphenyl phosphate (K_m 0·0030).

Slyke, 1922)). *B* represents the molar concentration of added NaOH and *C* the molar concentration of ester in the titration mixture at any given time. (A pK_1 and pK_2 for the pyrophosphoric esters could not be distinguished by this method.) The graphs for the titration are given in Fig. 1, and the pK values in Table 1.

Enzyme hydrolysis. A range of carbonate-veronal buffers covering pH 7·5–10·7, and a potent faecal phosphatase were prepared as described by Delory & King (1943), and their procedure for the determination of the pH optima and Michaelis constants was followed exactly. These results are shown graphically in Figs. 2 and 3.

DISCUSSION

It was expected, when these two substituted phenyl phosphates were made, that they would show a greater rate of hydrolysis than unsubstituted phenyl phosphate, due to the strong positive inductive, acid-strengthening effect of the carboxyl group. At the pH values used for enzymic hydrolysis, however, the group which is influencing the secondary phosphate dissociation is the negatively charged ionized carboxyl group (—COO^-); and the two negative charges on the dissociated primary phosphate and carboxyl groups have outweighed the positive inductive action which we had expected.

These results, however, agree with those of Delory & King (1943) in showing an increase in the amount of hydrolysis and a shift of the pH optimum to a more alkaline reaction with increasing acidity of the substrate.

SUMMARY

1. *o*-Carbomethoxyphenyl pyrophosphate and *p*-carbethoxyphenyl pyrophosphate have been prepared; they are converted by mild hydrolysis to the orthophosphoric esters *o*-carboxy- and *p*-carboxyphenyl phosphate.

2. These esters are not such strong acids (pK *o*-, 6·11; *p*-, 6·40) as unsubstituted phenyl phosphate (pK 5·73).

3. The optimum pH values for enzymic hydrolysis by faecal phosphatase (*o*-, 9·10; *p*-, 8·00) are not as alkaline as that of phenyl phosphate (9·73). The rates of enzymic hydrolysis are also less.

4. The Michaelis constants (K_m for *o*-, 0·0010; *p*-, 0·0030) are greater than that of phenyl phosphate (K_m 0·0006).

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The Fermentable Form of Fructose

By R. H. HOPKINS and M. HORWOOD
Department of Industrial Fermentation, University of Birmingham

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Hopkins (1931) found that when a rapid fermentation of fructose by brewer's yeast is arrested the residual sugar exhibits mutarotation in the positive sense. From this and other observations it was concluded that one form of fructose was specifically fermented or at least preferred, that this form possessed a specific rotation less negative than that of fructose at equilibrium, and was fructofuranose. It was not possible to furnish clear experimental proof since neither α-fructopyranose nor any form of fructofuranose could be isolated in the crystalline form. However, a preparation of fructose exhibiting substantially less negative specific rotation than that of the solution at equilibrium can be prepared by slowly fusing normal fructose and cooling suddenly. The rates of fermentation of such preparations have been compared with those of fructose at equilibrium, and of freshly dissolved normal fructose.

MATERIALS AND METHODS

Substrates. D-Fructose recrystallized from ethanol (Harding, 1922), was heated slowly with stirring in a nickel crucible immersed in a glycerine bath until the temperatures stated in Table 1 were attained. The crucible was then plunged into solid CO_2. It is important to minimize access of moisture to the sugar throughout.

Fermentation. Brewer's top fermentation yeast was used. Rates of fermentation were measured as by Hopkins & Roberts (1935*a*). In the fermentation vessel the brewer's yeast in suspension was brought to the desired temperature and the sugar, in solid form or in solution at equilibrium, was

contained in a small beaker on a float on the surface. This sugar was discharged into the yeast suspension by starting the shaker.

RESULTS

Table 1 shows the initial specific rotation at 20° of the fructose preparations obtained by heating.

Table 1. *Effect of heat on specific optical rotation of* D*-fructose*

Temperature to which fructose was heated (°)	Colour of product	$[\alpha]_D^{20°}$ Before mutarotation (°)	$[\alpha]_D^{20°}$ After mutarotation (°)
Unheated	White	—	−88·9
110	White	−59·8	−88·7
120	White	−54·6	−88·7
130	Cream	−56·1	−83·7
140	Buff	−57·0	−76·4

These values were obtained by extrapolation from a succession of polarimetric readings. If $[\alpha]_D^{20°}$ of β-fructopyranose is $-131°$ and that of β-fructofuranose is $+17°$ (Bailey & Hopkins, 1933), then $[\alpha]_D^{20°} - 54·6°$ would correspond to 51 % of β-fructofuranose. It was decided to adopt 120° as the maximum temperature of heating. As the value of $[\alpha]_D^{20°}$ before mutarotation was found to increase in a few hours even when the heated sugar was stored in a vacuum desiccator, it was necessary to prepare the specimens immediately before use.

give low induction periods, so that the maximum rates of fermentation were attained before mutarotation could be appreciable. Only the early rates are unaffected by the latter.

The preheated fructose, $[\alpha]_D^{20°} = -57$ to 58°, was much more rapidly fermented than the equilibrium solution and the latter than the β-fructopyranose form, especially at 35°. The fermentable component was apparently maintained, when equilibrium solution and β-fructopyranose were used, by continual transformation of β-fructopyranose into fructofuranose with accompanying mutarotation. (Incidentally the temperature coefficients $v_{35°}/v_{25°}$ of the maximum velocities agree with that of the mutarotation of fructose. For fuller treatment, see Hopkins & Roberts, 1935*b*.) With the heated fructose, however, mutarotation, instead of reinforcing it, acted against the fermentation, which in consequence slowed down markedly after attaining its maximum rate, especially at 35°.

In these experiments an attempt was made to approach as nearly as possible to the use of pure fructofuranose. Gottschalk (1943) employed conditions designed to minimize the quantity of fructofuranose present. He found that if β-fructopyranose was added to a yeast suspension at 0° the rate of fermentation was very small compared with that of glucose under the same conditions, and was independent of yeast concentration. The rate was

Table 2. *Rate of fermentation of forms of fructose*

(Fructose 0·5 g.; yeast 10 g.; pH 4·6, 0·01 N-acetate buffer; 50 ml.)

Time (min.) ...	2	3	4	5	6	7	8	9	10	15
	Temperature 35°; CO_2 (mg./min.)									
β-Fructopyranose	8·1	13·9	16·2	15·7	14·4	12·9	11·6	11·1	10·4	6·4
Equilibrium fructose	16·4	18·4	16·8	15·1	13·5	11·9	10·3	9·5	9·0	6·0
Preheated fructose, $[\alpha]_D^{20°} - 56·5°$	22·2	25·2	17·3	10·9	10·7	10·3	9·3	8·5	7·6	4·7
	Temperature 25°; CO_2 (mg./min.)									
β-Fructopyranose	2·8	4·3	6·0	7·1	7·4	7·1	6·7	6·2	6·2	4·8
Equilibrium fructose	4·5	7·1	8·9	9·3	8·9	8·4	7·8	6·9	6·4	4·9
Preheated fructose, $[\alpha]_D^{20°} - 58·5°$	5·3	8·1	9·7	9·6	9·2	8·9	8·0	6·9	6·4	4·2

Fermentations performed using β-fructopyranose, equilibrium and preheated fructose are summarized in Table 2.

DISCUSSION

The rate of fermentation of fructose is appreciably influenced by concentration of the sugar over the range 0–2 %, especially if a large proportion of yeast is used. Yeast and temperatures were selected to indeed approximately that of the conversion of the pyranose into the furanose form by mutarotation.

The results reported here, like those of Gottschalk (1943, 1945, 1947) and of Suomalainen & Toivonen (1948), support the hypothesis first put forward by Hopkins (1931), and strongly supported by Gottschalk, that fructofuranose is the only form of fructose fermentable by yeast.

SUMMARY

1. Dried crystalline D-fructose, heated to 120° and cooled suddenly, yielded a product which, it is claimed, contained 51 % of fructofuranose.

2. This product was fermented much more rapidly than fructose at equilibrium in a corresponding solution.

3. This observation supports the theory that fructofuranose is the only form of fructose fermentable by yeast.

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The Biogenesis of Porphyrins

2. THE ORIGIN OF THE METHYNE CARBON ATOMS

BY HELEN M. MUIR AND A. NEUBERGER

The National Institute for Medical Research, Hampstead, London, N.W. 3

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The method which the organism uses to synthesize protoporphyrin has aroused much speculation (Dobriner & Rhoads, 1928, 1940; Rimington, 1938; Turner, 1940). No experimental evidence for the mechanism of porphyrin synthesis was obtained, however, until Bloch & Rittenberg (1945) reported that deuterium, when supplied to rats in the form of acetate, was found in their blood haem. Shemin & Rittenberg (1946*a*, *b*) showed that the blood porphyrin from men and rats who had received ^{15}N-labelled glycine had a relatively high isotope content. Other nitrogen-labelled compounds including ammonia, leucine, proline, glutamic acid, histidine (Tesar & Rittenberg, 1947), aspartic acid (Wu & Rittenberg, 1949), and ethanolamine (Muir & Neuberger, 1949*a*) were relatively ineffective in rats and rabbits respectively.

In a previous paper (Muir & Neuberger, 1949*a*), the partial degradation of mesoporphyrin on a small scale was described. Three products of oxidation were isolated, viz. carbon dioxide, which was derived mainly from the methyne carbon atoms, methylethylmaleic acid imide from the non-acidic rings I and II of the porphyrin, and 1-methyl-2:2′-carboxyethylmaleic acid imide (haematinic acid), from the acidic rings III and IV. It was shown that feeding ^{15}N-labelled glycine to rabbits produced a blood porphyrin in which the isotope content was the same in the acidic and non-acidic fractions. Wittenberg & Shemin (1949) independently carried out an analogous degradation of the haemin obtained from a human subject and from ducks who had received ^{15}N-labelled glycine, and obtained the same result.

In the present investigation the method has been used to find the distribution within the protoporphyrin molecule of labelled carbon atoms derived from likely precursors. These included sodium acetate, glycine, serine and sodium formate labelled with ^{14}C and ^{15}N as indicated by the following formulae:

$^{14}CH_3.COONa$, $CH_3.{}^{14}COONa$,
$NH_2.{}^{14}CH_2.COOH$, $^{15}NH_2.{}^{14}CH_2.COOH$,
$^{14}CH_2OH.CH(NH_2).COOH$ and $H^{14}COONa$.

For preliminary communication see Muir & Neuberger (1949*b*).

EXPERIMENTAL

Treatment of animals and administration of compounds

Rabbits. Male and female Hollingsworth half-lopped rabbits were used (2·5–3 kg. body weight). They were given the Institute stock diet, except in the experiment in which glycine was administered. In this experiment the animal was kept on a low protein diet of bran, hay and cabbage during the experiment, and for 1 month prior to it. Except in the experiment with $^{14}CH_3.COONa$, to increase the amount of isotope incorporated into the haem, an anaemia was produced by withdrawing a total of 50–70 ml. of blood over 2–4 days. The labelled compound was injected sub-

cutaneously on the day following the last bleeding. The animals were killed from 3–9 days after the injection. They were anaesthetized with ether, and the blood was collected from the aorta, while heparin-containing saline was perfused through the vena cava.

Rats. Albino rats of the Institute stock (250–300 g. body weight) were used for two experiments in which serine and formate were given. These animals were also being used for other experiments. No anaemia was produced. In the serine experiment the rats received Na benzoate and γ-phenyl-α-aminobutyric acid as well as serine, and in the formate experiment the animal was given glycine and Na benzoate as well as Na formate. The labelled compounds were mixed with the food, and were not injected.

Isotopic compounds

^{14}C used in these experiments was obtained from the U.S. Atomic Energy Commission. The two ^{14}C-labelled acetates were synthesized by the Radiochemical Centre, Amersham, Buckinghamshire. $NH_2.{}^{14}CH_2COOH$ and $H^{14}.COOH$ were supplied by Dr H. R. V. Arnstein, who also synthesized the $^{14}CH_2OH.CH(NH_2).COOH$ (Arnstein, 1949). The ^{15}N-labelled glycine was made by the method of Schoenheimer & Ratner (1939), from isotopic NH_4NO_3.

Isolation of mesoporphyrin

The haemin from washed erythrocytes was crystallized, converted to protoporphyrin methyl ester and then reduced to mesoporphyrin as described by Muir & Neuberger (1949*a*). To obtain larger quantities for degradation, the haemin was generally diluted with an appropriate quantity of inactive haemin, before being converted to mesoporphyrin.

Degradation of mesoporphyrin with CrO_3

Apparatus. The oxidation was carried out in a Kjeldahl apparatus. N_2, washed free of CO_2, was introduced through the inlet tube, the side arm of which carried a short burette attached by a rubber connexion to a right-angle tube leading to an absorption train. Thus the flask could be shaken during the reaction. The absorption train contained three units, each of which consisted of a tapered pyrex centrifuge tube (11 cm. long, 1·2 cm. internal diameter) previously weighed, containing 3·905 N-NaOH free of CO_2. Each tube had a B 19 cone joint at the top, which fitted into a socket carrying an inlet tube reaching to within 1 cm. of the bottom of the tube. The socket also carried an outlet tube which connected with the next absorption unit through a B 10 joint. Both inlet and outlet tubes were fitted with taps, so that each unit could be shut off in turn. The whole apparatus could be dismantled for cleaning.

Oxidation. The mesoporphyrin methyl ester was first dissolved in 50% (v/v) H_2SO_4, then cooled to 0° while the CrO_3 was added from the burette in small quantities over 1 hr., with occasional shaking. An excess of 10–12% of available O was used. After 5–6 hr. the unused O was estimated iodometrically, and the imides extracted from the reaction mixture with ethyl acetate. The separation and purification of the imides was performed as described by Muir & Neuberger (1949*a*).

Isolation and estimation of CO_2. The CO_2 was carried over in a stream of N_2. At the end of the reaction each absorption unit was disconnected in turn, the tip of the inlet tube washed with CO_2-free water, and an excess of saturated $BaCl_2$ added. The tube was capped at once, and centrifuged. No $BaCO_3$ was ever obtained in the third tube. The $BaCO_3$ was resuspended in CO_2-free water, and washed on the centrifuge until free of Na^+ and Cl^-. It was finally washed with ethanol and ether, dried *in vacuo* at 100° and the centrifuge tube weighed again. The original supernatant and the washings from the $BaCO_3$ precipitation were combined, made up to volume, and the unused NaOH estimated by titration. Thus the CO_2 was estimated both volumetrically and gravimetrically. The values agreed to within 3%. An experiment in which CO_2 was liberated from 250·7 mg. of pure $NaHCO_3$ with H_2SO_4 showed that CO_2 could be recovered quantitatively by this method.

Isotope determinations

^{15}N was estimated with the mass spectrometer, and ^{14}C was determined with a bell-shaped He-filled Geiger-Müller counter, having a thin mica window. This counter has an unusually low background count of 10–11 counts/min. The radioactivity measurements were carried out on solid samples placed on disks of identical geometry. The material was spread evenly by the pellet technique (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949; Popják & Beeckmans, 1950). The layer of material was 'infinitely thick', i.e. not less than 25 mg./sq. cm. Under such conditions the number of counts is linearly proportional to the specific activity. No correction for self-absorption was made. When $BaCO_3$ samples were compared with samples of organic material, a back-scattering correction of 4% was made. This was determined experimentally as recommended by Calvin *et al.* (1949).

In order to compare the radioactivities of compounds of different molecular weight, the counts/min./mg. sample were multiplied by the mol.wt. $\times 10^{-3}$ of the substance. These values are designated counts/min./mmol. Standard errors were calculated as described by Calvin *et al.* (1949). Some disk samples were counted on a windowless proportional counter, which had greater sensitivity.

Conversion of haemin to deuterohaemin

Haemin (70 mg.) was converted to deuterohaemin by heating with resorcinol (Fischer & Hummel, 1929). (Found: Cl 5·9. Calc. for $C_{30}H_{28}O_4N_4FeCl$: Cl 5·9%.)

RESULTS

Particulars of conditions used in the various experiments are summarized in Table 1.

Degradation of mesoporphyrin

In a previous paper (Muir & Neuberger, 1949*a*) it was stressed that a knowledge of the yields in the oxidation is important for the interpretation of degradation experiments involving tracers, and the belief was expressed that 80–90% of the carbon dioxide evolved comes from the four methyne carbon atoms, and the two carbon atoms of the methyl ester groups. In agreement with earlier results the yields of the two imides (Table 2) varied between 63 and 78%. The oxygen consumption varied from 93 to 102% of the theoretical, and the carbon dioxide

Table 1. *Details of biological experiments*

Exp. no.	Species	Body wt. (kg.)	Blood withdrawn (ml.)	Labelled compound injected: Formula	Wt. injected (mg.)	Isotopic dose (μc.)	Radioactivity of haemin (counts/min./mmol.)
1	Rabbit	3·9	0	$^{14}CH_3.COONa$	34·2	416	121·9±4·2
2	Rabbit	3·0	73	$CH_3.^{14}COONa$	29·8	363	83·4±2·6
3*a*	Rabbit	2·7	45	$^{15}NH_2.^{14}CH_2.COOH$	566·1	54	733·0±8·1
3*b*	Rat	0·302	0	$NH_2.^{14}CH_2.COOH$	9·6	9·6	138·8±4·2
4	Rats (2)	0·55	0	$^{14}CH_2.OH.CH(NH_2).COOH$	122	10	3·5±1·5
5	Rat	0·387	0	$H.^{14}COOH$	41·7	22·25	3·54±0·5*

* Estimated as protoporphyrin methyl ester.

production from 98 to 99 %. These findings indicate that the formation of the two imides is not quantitative, but that the side reactions which occur do not consist of extensive further oxidation of the two maleic imides.

Table 2. *Results of oxidation experiments*

(Particulars of animals used and compounds administered are given in Table 1.)

Exp. no.	Wt. of meso-porphyrin methyl ester (mg.)	Percentage of theoretical: O consumed	CO_2 produced	Wt. of crude imides obtained
1	269·7	93·0	68	67
2	246	100·1	99	78
3*a*	495·2	102	98·7	65
3*b*	100·1	99·5	97	63·5
4	33	98·5	70	—

Comparison of activities of haemin samples

Of all the substances tested, glycine produces by far the most active haemin (Table 1). Both serine and formate are relatively inefficient precursors. Carboxyl-labelled acetate is less efficient than methyl-labelled acetate, even though the rabbit used in the latter experiment was not anaemic. In a preliminary experiment with $CH_3.^{14}COOH$, the haemin obtained from a rabbit that was not anaemic had a very low activity. Radin, Rittenberg & Shemin (1949) have stated that the utilization of $^{14}CH_3.COOH$ is about six times more efficient, but it is not clear whether the experiments were carried out *in vivo* or *in vitro*.

Relative activities of degradation products

The experimental results are shown in Table 3. The proportions of the total activity of the original mesoporphyrin accounted for by the products are

Table 3. *Radioactivity of products of oxidation*

(Particulars of animals used and compounds administered are given in Table 1 and yields of products in Table 2. Results expressed as counts/min./mmol. —, means 'not determined'.)

Exp. no.	Compound injected	Mesoporphyrin methyl ester (*a*)	Haematinic acid (*b*)	Methylethylmaleic acid imide (*c*)	$BaCO_3$ (*d*)
1	$^{14}CH_3.COONa$	27·9 ±0·67	7·55±0·25	6·5 ±0·22	0·08
2	$CH_3.^{14}COONa$	32·35±1·09	12·66±0·31	2·97±0·15	0·44±0·3
3*a*	$^{15}NH_2.^{14}CH_2.COOH$	131·2 ±1·09	20·4 ±0·25	19·8 ±0·198	11·4 ±0·27
3*b*	$NH_2.^{14}CH_2.COOH$	30·45±0·65	3·88±0·12	—	—
		187·0 ±3·68*	—	—	14·73±0·79*
4	$^{14}CHOH.CH(NH_2).COOH$	11·07±3·3*	—	—	0

* Values obtained on proportional counter.

Table 4. *Comparison of activities of haematinic acid and methylethylmaleic acid imide*

(Letters *a*, *b*, *c* refer to molecular activities of mesoporphyrin, haematinic acid, and methylethylmaleic acid imide respectively. Results expressed as counts/min./mmol.)

Compound injected	$\frac{2b}{a}\times 100$	$\frac{2c}{a}\times 100$	$\frac{2(b+c)}{a}\times 100$	$\frac{b}{c}$
$^{14}CH_3.COONa$	54·1±4·36	46·6 ±4·0	100·7 ±6·3	1·16
$CH_3.^{14}COONa$	78·3±2·7	18·36±1·75	96·66±4·65	4·26
$^{15}NH_2\,^{14}CH_2.COOH$	31·1±2·06	30·2 ±1·74	61·3 ±2·69	1·03
			$\frac{4c}{a}\times 100$	
$NH_2\,^{14}CH_2.COOH$	—	25·5 ±4·2	51·0 ±8·4	—

given in Table 4. The assumption is made that the methylethylmaleic acid imide isolated represents equally both rings I and II, and the haematinic acid rings III and IV. Therefore the proportion of the activity which they represent is obtained by multiplying their molecular activities by two.

The molecular activity of the two imides was almost equal after injecting $^{14}CH_3.COONa$ and $^{15}NH_2.^{14}CH_2.COOH$. But after injecting $CH_3.^{14}COOH$, the molecular activity of the haematinic acid was more than four times that of the other imide.

Origin of methyne carbon atoms

The activity of the methyne carbon atoms has been determined in two ways. The activity of the carbon dioxide isolated in the oxidation is a direct measure of the activity of the methyne carbon atoms, but the accuracy is reduced, owing to side reactions discussed above. Moreover, the carbonate fraction contains carbon dioxide derived from the methyl ester groups, and it is assumed that these are quantitatively oxidized. Since six molecules of carbon dioxide are produced from one molecule of mesoporphyrin ester, the molecular activity of the barium carbonate is multiplied by six. The figures given in Table 5 show clearly that in the two glycine experiments, the barium carbonate fraction accounts for approximately 50 % of the total activity of the mesoporphyrin, but after giving either of the acetates or serine, the activity was negligible.

The relative activity of the methyne carbon atoms has also been calculated by deducting the proportion of the activity due to the two imides (Table 4, column 4), from the activity of the original mesoporphyrin. These values are given in Table 5. This calculation shows that with both acetates the imides account for the whole of the activity of the mesoporphyrin. In agreement with the barium carbonate results this shows that neither of the two carbon atoms of acetate contributes to any appreciable extent to the formation of the methyne bridges. On the other hand, with glycine, the imides appear to account for only 50–60 % of the activity of the porphyrin. Thus, in the first glycine experiment, the calculated value for the relative activity of the methyne carbon atoms was $38{\cdot}7 \pm 2{\cdot}8$ % of the total. In the second experiment only one imide was isolated, and it was assumed that the molecular activities of the two imides were equal (Table 4). On this basis, the relative calculated activity of the methyne carbon atoms was found to be 49 %. The relative molecular activity of the barium carbonate fraction in the two experiments were $50{\cdot}0 \pm 2{\cdot}1$ and $45{\cdot}9 \pm 5{\cdot}9$ % respectively.

Although the agreement between the values obtained in the various experiments is only moderately good, nevertheless it seems justified to conclude that (*a*) of all the compounds tested methylene-labelled glycine alone contributes significantly to the formation of the methyne bridges; (*b*) that the methyne bridges account for 40–50 %, and the pyrrole rings 50–60 % of the total activity of the porphyrin obtained after glycine administration.

Comparison of isotope ratios of doubly labelled glycine and haemin

From the results given in Table 6, it can be calculated that the dilution of ^{15}N, i.e. the ratio of ^{15}N (atom % excess) of the glycine administered to that of the haemin is 82·5.

To compare the two dilution factors, the ^{14}C results are given in counts/min./100 mg. N. The ^{14}C dilution factor, as calculated from the results of Table 6, is 38·02. The ratio of the two dilution factors will give the relative proportions of labelled carbon and nitrogen atoms entering the porphyrin molecule. This ratio is 2·17, i.e. 2·17 methylene carbon atoms are used for porphyrin synthesis for each nitrogen atom derived from the same amino-acid.

Table 5. *Comparison of activities of methyne carbon atoms as calculated by difference and as found in carbonate fraction*

(Letters *a*, *b*, *c*, *d* refer to molecular activities of mesoporphyrin, haematinic acid, and methylethylmaleic acid imide and barium carbonate respectively. Results expressed as counts/min./mmol.)

Compound injected	$\frac{a-2(b+c)}{a} \times 100$	$\frac{a-4c}{a} \times 100$	$\frac{6d}{a} \times 100$
$^{14}CH_3.COONa$	$-0{\cdot}3 \pm 6{\cdot}3$	—	0
$CH_3.^{14}COONa$	$3{\cdot}34 \pm 4{\cdot}65$	—	0
$^{15}NH_2.^{14}CH_2.COOH$	$38{\cdot}7 \pm 2{\cdot}82$	$39{\cdot}6 \pm 3{\cdot}58$	$50{\cdot}02 \pm 2{\cdot}13$
$NH_2.^{14}CH_2.COOH$	—	$49{\cdot}0 \pm 8{\cdot}7$	$45{\cdot}9 \pm 5{\cdot}9$

Table 6. *Comparison of ^{15}N and ^{14}C contents of glycine and haemin*

	^{15}N (atom % excess)	^{14}C (counts/min./100 mg. N)
Glycine	28·7	$50{\cdot}06 \times 10^6$
Haemin	0·348	$1{\cdot}317 \times 10^{6*}$

* No correction for back scattering.

Comparison of activity of haemin and deuterohaemin

When haemin is converted to deuterohaemin, two vinyl groups of rings I and II are lost. The relative molecular activity of the two compounds will give a measure of the radioactivity of the four carbon atoms removed. Table 7 shows that, after injecting $CH_3.{}^{14}COONa$, 7% of the activity is lost with the vinyl groups. This value is probably statistically significant and suggests that the carboxyl carbon atom of acetate is utilized to some extent for the formation of the vinyl groups of protoporphyrin. After injecting $^{14}CH_3.COONa$ the loss of activity was 23·2%. It is concluded that the methyl carbon atom of acetate is used to a greater extent than the carboxyl carbon atom, for the formation of the vinyl groups.

Table 7. *Comparison of radioactivity of haemin*

(Results expressed as counts/min./mmol.)

Compound injected	Haemin (A)	Deuterohaemin (B)	$B/A \times 100$
$^{14}CH_3.COONa$	121·9±4·2	93·6±3·6	76·8±5·15
$CH_3.{}^{14}COONa$	83·4±2·6	77·5±2·4	93·0±4·4

DISCUSSION

The origin of the methyne carbon atoms

Previous work has already shown that glycine is a biological precursor of protoporphyrin; the nitrogen atom (Shemin & Rittenberg, 1946*a*, *b*), and the methylene carbon atom (Altman, Casarett, Masters, Noonan & Salomon, 1948), but not the carboxyl carbon atom (Grinstein, Kamen, & Moore 1948) are used for the synthesis of the porphyrin. The experiments described in this paper demonstrate that the methylene carbon atom is the source of at least some of the methyne bridge atoms of protoporphyrin. The specificity of glycine in this respect is indicated by the observation that neither of the two acetates, nor formate nor serine produces significant activity in the methyne carbon fraction. Bufton, Bentley & Rimington (1949) have also found that formate is not utilized for the synthesis of haem by fowl erythrocytes *in vitro*. The hydroxymethyl group of serine is probably a precursor of the metabolic 'one-carbon' fragment (Sakami, 1949*b*) and the negative results obtained with serine and formate suggest that the methyne carbon atoms are not derived directly from such a 'one-carbon' fragment. These results also refute the hypothesis that, because glycine can be converted to serine (Sakami, 1948, 1949*a*; Siekevitz & Greenberg, 1949; Goldsworthy, Winnick & Greenberg, 1949), serine and not glycine is the immediate porphyrin precursor.

As already shown (Muir & Neuberger, 1949*a*; Wittenberg & Shemin, 1949), glycine must provide at least two of the four nitrogen atoms of protoporphyrin. However, as no other nitrogenous precursor has yet been found, amongst numerous compounds tested, it is likely that all four nitrogen atoms of the porphyrin are derived from glycine. This is supported by the observation that the newly formed porphyrins of a congenital porphyric have a higher ^{15}N content even than the glycine excreted as hippuric acid (Gray & Neuberger, 1950).

By using $^{15}NH_2.{}^{14}CH_2.COOH$, it was found that at least twice as many labelled carbon as nitrogen atoms appear in protoporphyrin. This ratio of 2 : 1 cannot be explained by loss of ^{15}N from doubly labelled glycine followed by reamination, because such a reversible deamination does not occur to a significant extent (Shemin, 1946, Elliott & Neuberger, 1950; Arnstein & Neuberger, unpublished observations). It would therefore appear that for each glycine molecule which supplies both nitrogen and methylene carbon atoms, there is approximately one additional molecule, which provides only the methylene carbon atom for the synthesis of protoporphyrin. As all four nitrogen atoms of the porphyrin appear to be derived from glycine, it must follow that at least eight carbon atoms are also derived from this source. The actual number of labelled carbon atoms calculated on this basis from the isotope ratio of 2·17, is 8·68. If all the four methyne carbon atoms are derived from glycine, it would follow that there are 4·68 labelled atoms in the pyrrole rings. In support of this deduction, the relative activity of the rings, as determined by degradation experiments, was found to be greater than that of the methyne bridges. The ratio was 61·3/38·7 in the first glycine experiment in rabbits. However, by calculation from the $^{14}C/^{15}N$ ratio, the ratio of activity of the pyrrole rings to that of the methyne bridges should be 54/46. It might therefore be argued that only three methyne carbon atoms are derived from glycine, in which case there should be 5·68 labelled atoms in the rings, resulting in a ratio of activities of 65·5/34·5. The accuracy of the results does not justify a decision being made. Nevertheless, in the second glycine experiment in rats, the ratio of activity of the ring to that of the methyne carbon atoms, was found to be 51/49, which would support the supposition that there are an equal number of labelled atoms in the rings and methyne bridges.

The $^{14}C/^{15}N$ ratio is not a whole number and leads to a calculated value of 8·68 labelled carbon atoms

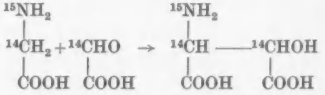
for the molecule. If eight are derived directly from glycine, the remaining 0·68 might represent the aggregate non-specific labelling of the molecule. Sprinson (1949) has shown that the methylene group of glycine is converted to an appreciable extent to both the methyl and carboxyl carbon atoms of acetate. If this conversion occurred to any extent in the bone marrow, then the acetate immediately available for porphyrin synthesis would be appreciably radioactive, because it would not be diluted by the total acetate of the body. Furthermore, if the non-specific labelling is due to the secondary conversion of glycine to acetate, the additional activity should appear in the pyrrole rings of the porphyrin, and not in the methyne carbon atoms, in agreement with the experimental results. The difference between the ratios of activities of the rings/methyne bridges, in the two glycine experiments with rabbits and rats could be explained by a variation in the non-specific labelling in the two species. Taking into account the effect of non-specific labelling, it would appear that the carbon atoms directly derived from glycine are equally distributed over the rings and methyne bridges. If it is assumed that all four nitrogen atoms are derived from glycine, it would then follow that all four methyne carbon atoms are also formed from glycine.

Distribution of labelled carbon atoms in the pyrrole rings

After administration of glycine and methyl-labelled acetate, the two imides were found to have almost equal molecular activities. It follows that the activity which derives from these two precursors is equally distributed between rings I + II and rings III + IV. On the other hand, the haematinic acid obtained from the experiment with carboxyl-labelled acetate, is more than four times as active as the methylethylmaleic acid imide. Presumably the radioactivity is due largely to the carboxyl group, which is what distinguishes the haematinic acid from the other imide. Radin *et al.* (1949) have also stated that the carboxyl group of acetate gives rise to the carboxyl groups of protoporphyrin. The haemin obtained after injecting methyl-labelled acetate lost almost 25 % of its activity when it was converted to deuterohaemin. At least one of the two carbon atoms of the vinyl groups must therefore be formed from the methyl group of acetate. It has recently been shown, with the use of deuterium-labelled acetate, that about one-third of the hydrogen atoms of haemin are derived from the hydrogen atoms of acetate (Ponticorvo, Rittenberg & Bloch, 1949). Some of the carbon atoms of other side chains besides the vinyl groups must therefore also be formed from the methyl group of acetate. The haemin from carboxyl-labelled acetate lost only 7 % of its activity when it was converted to deuterohaemin, which suggests that the carboxyl group of acetate is only used to a slight extent for the formation of the vinyl side chains.

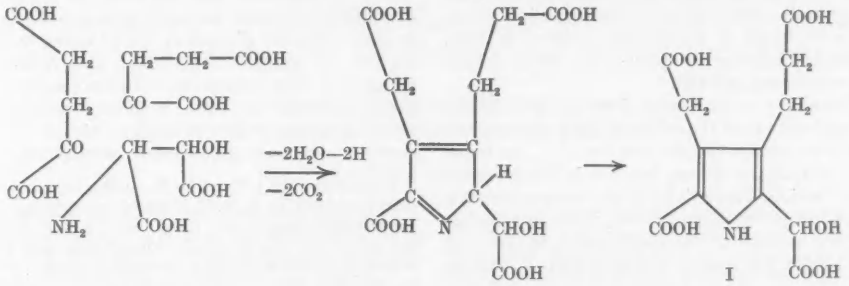
A suggested mechanism of porphyrin synthesis

The experimental results have shown that the methyne carbon atoms of protoporphyrin are largely, if not exclusively, derived from the methylene carbon atom of glycine, and that the amino-acid provides probably eight methylene carbon atoms and four nitrogen atoms for the synthesis. There is no reason to suppose that the N—C bond of the glycine molecule supplying the nitrogen is ever broken, so that the methylene carbon atom of glycine provides, presumably, one of the two carbon atoms of each pyrrole ring in the porphyrin molecule, in addition to the adjacent bridge carbon atoms. On the basis of these results, it is postulated that in the process of porphyrin synthesis, two molecules of glycine condense with loss of one nitrogen atom to a four-carbon compound. The four-carbon compound must subsequently lose its two carboxyl groups, because the carboxyl groups of glycine are not utilized (Grinstein *et al.* 1948). The nitrogen of glycine is utilized very efficiently, and therefore in any postulated intermediate the C—N link should have a metabolic stability similar to that in glycine. The four-carbon fragment is therefore probably not aspartic acid, because Wu & Rittenberg (1949) showed that ^{15}N-labelled aspartic acid loses its label too rapidly to be demonstrated as a nitrogenous precursor of protoporphyrin. However, Ratner, Nocito & Green (1944) have isolated a glycine oxidase from various species of animals, and have shown that this enzyme produces glyoxylic acid from glycine. Glyoxylic acid might therefore be involved in the formation of the four-carbon compound, which could be either hydroxyaspartic acid, or the related dehydration compound aminofumaric acid.



It is reasonable to suppose that the pyrrole rings are built up by the condensation of the four-carbon fragment with one of the intermediates of the Krebs cycle. It has been suggested by Lemberg & Legge (1949) and Lemberg (private communication), that α-ketoglutarate might be the compound involved. On this basis, the utilization of acetate must occur by its entry into the tricarboxylic acid cycle. Slightly modifying Lemberg's suggestion it is proposed that the four-carbon compound derived from glycine condenses with two molecules of α-keto-

glutarate to form a substituted pyrrole-2-carboxylic acid (I).



The participation of ketoglutarate in this way is consistent with most of the results of this paper. The greater use of the methyl than the carboxyl group of acetate for porphyrin synthesis is explained, since entry of carboxyl-labelled acetate into the cycle by any of the mechanisms believed to occur in the animal organism (for reviews see Krebs, 1943; Wood, 1946; Buchanan & Hastings, 1946; Bloch, 1947) would produce α-ketoglutarate labelled only on the carboxyl groups. The suggested mechanism for the formation of the pyrrole and the subsequent synthesis of porphyrin must eliminate most of the labelled carboxyl groups. On the other hand, the entry of methyl-labelled acetate into the cycle would at first lead to labelling of the ketoglutarate on one of the methylene carbon atoms, to be followed by a general redistribution over all the other carbon atoms as the cycle is repeated. In the synthetic reactions discussed above, only a small proportion of the activity due to the methyl group of acetate would thus be lost. This hypothesis would also explain why the haematinic acid fraction obtained after administration of carboxyl-labelled acetate is considerably more active than the methylethylmaleic acid imide. However, the fact that the latter has any activity at all suggests that there is a mechanism, as yet undiscovered, by which the carboxyl carbon atom of acetate is transformed to the methylene or carbonyl atoms of ketoglutarate. Radin *et al.* (1949) have also reported that in the haem obtained from bird red cells incubated with carboxyl-labelled acetate, only half of the total activity is due to the carboxyl groups. The formation of the pyrrole (I) by the proposed mechanism would explain the finding that at least one of the vinyl carbon atoms is derived from the methyl-carbon atom of acetate, and it would also explain the observation of Ponticorvo *et al.* (1949) that deuterium supplied as acetate is found in haem. Since acetate does not form the methyne groups, the methyne hydrogen atoms cannot come from this source, and therefore the deuterium must be situated in the side-chains. This agrees with the suggested mechanism, which implies that at least one carbon atom in each of the eight side chains of protoporphyrin is derived from the methyl carbon atom of acetate.

Rimington (1949) has pointed out that an attractive analogy for the biological synthesis of porphyrins exists in a chemical synthesis from substituted 5-hydroxymethylpyrrole-2-carboxylic acids, which readily undergo self-condensation to form porphyrins by decarboxylation and oxidation (Siedel & Winkler, 1943). Self-condensation of the pyrrole (I) by the Siedel-Winkler reaction would produce a uroporphyrin, which would finally lose all but two of its carboxyl groups when it is converted to protoporphyrin. This reaction would explain the fact that none of the carboxyl groups of the glycine molecules, and only a small number of the carboxyl groups of the acetate molecules which are used for the synthesis of the porphyrin survive in the final product. In the conversion of the pyrrole (I) to protoporphyrin there must also be a dehydrogenation, involving loss of four hydrogen atoms/protoporphyrin molecule. At what stage this occurs remains obscure.

This mechanism fully explains the formation of porphyrins of series I. In the formation of porphyrins of series III, however, it would have to be assumed that one of the rings, probably ring IV is formed by a somewhat different mechanism leading to an inverted arrangement of the side chains.

SUMMARY

1. Porphyrin synthesis in rabbits and rats was investigated after administration of sodium acetate, glycine, serine and formic acid labelled as indicated in the following formulae:

$^{14}CH_3.COONa$, $CH_3.^{14}COONa$,
$NH_2.^{14}CH_2.COOH$, $^{15}NH_2.^{14}CH_2.COOH$,
$^{14}CH_2OH.CH(NH_2).COOH$, $H.^{14}COOH$.

The porphyrin was degraded by oxidation to carbon dioxide, methylethylmaleic acid imide and haematinic acid.

2. The results showed that just over twice as many carbon atoms derived from the methylene group of glycine were incorporated as nitrogen atoms from the same amino-acid. About half the labelled carbon atoms were found in the methyne carbon fraction, whilst approximately one-quarter was found in each of the two imides isolated.

3. The methyl carbon atom of acetate was utilized more efficiently than the carboxyl carbon atom, and the activity of the former was found to an equal extent in both the imides, but not in the methyne carbon fraction. The activity of the carboxyl carbon atom of acetate was found chiefly in the haematinic acid. One or both of the vinyl carbon atoms were derived from the methyl carbon atom of acetate. Serine and formate were not utilized to any significant extent.

4. It is suggested that one molecule of glycine is oxidized to glyoxylic acid and then condenses with another molecule of glycine to give either hydroxyaspartic or aminofumaric acid. This four-carbon compound then condenses with two molecules of α-ketoglutarate to form a highly carboxylated pyrrole, which by further condensation with loss of carbon dioxide could give rise to porphyrins.

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The Preparation of Adenosinetriphosphate from Acetone-dried Muscle and a Chemical Method for the Preparation of Adenosinediphosphate

By MARIANNE BIELSCHOWSKY*
Department of Pathology, University of Sheffield

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During recent years adenosinetriphosphate has been shown to take part in an ever-increasing number of biochemical processes. It may therefore be worth while to record some findings on the isolation of adenosinetriphosphate from acetone-dried muscle and on some properties of its magnesium salt, which allow the preparation of adenosinediphosphate by chemical means.

In a preliminary communication Bielschowsky & Green (1943) gave a short account of the identification of the active principle present in a muscle preparation, 'myotoxin', described by Dyckerhoff, Schörcher & Torres (1939). Adenosinetriphosphate was found to be the agent responsible for the toxic action of myotoxin.

EXPERIMENTAL AND RESULTS

Analytical methods

Ribose was estimated by the method of Mejbaum (1939). The colorimetric readings were taken with an Ilford red filter and standard grey screen (King, Haslewood & Delory, 1937), calibrated against standard solutions of pure arabinose. The use of this procedure allowed accurate readings with a simple colorimeter (Klett), even when very small amounts of pentose had to be estimated. Lohmann's (1928) method of hydrolysis was used in the estimation of total, 7 min. and inorganic P by Briggs's method (1922). Nitrogen estimations were performed by a semi-micro-Kjeldahl method. Digestion was carried out with the addition of pure glucose and Cu selenite (Myrbäck & Euler, 1931). Ba was estimated as sulphate, Mg according to Tisdall & Kramer (1921). C and H estimations were carried out by Dr Weiler and Dr Strauss, Oxford.

Preparation of Dyckerhoff's 'myotoxin'

The myotoxin was obtained from rat, rabbit or ox muscle in the following way: muscle removed immediately after the death of the animal was minced with the greatest possible speed into five to seven times its weight of cold acetone with constant stirring. This process was completed within 10 min. of the death of the animal. The muscle mince was strained through muslin and resuspended in 7 vol. of fresh acetone. After removal of the acetone the process was repeated once more using 5 vol. of acetone. The muscle residue was then shaken with 3 vol. of ether which was filtered off with suction. The material was washed with 2 vol. of ether on the Büchner funnel, and after most of the ether had been removed, the residue was spread out on filter paper and the remaining ether allowed to evaporate at room temperature. The last traces were removed in a vacuum desiccator over $CaCl_2$. Finally the material was powdered, avoiding heating during this process. The dried muscle powder was stored in the refrigerator, where it seemed to remain stable for several months, and was used when required.

The muscle powder (250 g.) was thoroughly mixed with 1250 ml. of saline, extracted for 7 min. at room temperature on a shaker and immediately pressed through a cloth of fine texture. The extract was heated quickly and kept boiling for 7 min. It was then rapidly cooled to room temperature and the precipitate was removed by filtration (Whatman paper no. 54) and discarded. The clear filtrate was transferred into large measuring cylinders, placed in an ice bath, and 3 vol. of ice-cold methanol were added with stirring. After standing for 2 hr. the clear supernatant fluids were removed. The remaining suspension was spun down and resuspended in the centrifuge tubes with approximately 2 vol. of methanol and again centrifuged. The washing with methanol was repeated and followed by two washings with absolute ether. The white precipitate was transferred into wide capsules, quickly broken up and the ether evaporated in a vacuum desiccator (yield 3·25–3·6 g.).

Batches of 5 g. of this product were placed in a mortar and dissolved in 250 ml. of boiling saline, added in small portions. The solution was transferred to a fairly large beaker and boiled for 3 min. After rapid cooling to room temperature it was filtered (Whatman paper no. 5). After adding 3 vol. of cold methanol to the filtrate, it was left in the refrigerator for 2 hr. or longer. The resulting precipitate was washed and dried as described above. A white, amorphous, non-hygroscopic powder, soluble in water, was obtained which corresponds to 'myotoxin' (yield from 250 g. dry muscle powder, 2·3–2·5 g.).

The yield of this product can be increased by about 50% if the muscle residue after the saline extraction is re-extracted with about 600 ml. of saline and the two extracts pooled and worked up as described. Preparations different in appearance and properties from those obtained by cold saline extraction resulted from re-extraction with boiling saline as recommended by Dyckerhoff *et al.* (1939). The largest amount of muscle powder which could conveniently be worked up at one time in a laboratory with limited facilities was 250 g.

* Present address: Department of Cancer Research, University of Otago Medical School, Dunedin, New Zealand.

Chemical examination of the white powder (myotoxin) showed that it contained 15–20% protein and 30% ash, the latter consisting mainly of magnesium pyrophosphate. Only traces of potassium and sometimes of glycogen were found. The orcinol reaction was strongly positive.

Isolation of adenosinetriphosphate from myotoxin

Separation of the protein from the non-protein components present in myotoxin was achieved by salicylic acid or 2N-hydrochloric acid.

To 1 g. of the preparation, dissolved in 50 ml. of water, a saturated aqueous solution of salicylic acid was added with stirring and ice cooling until the pH of the mixture was 4·5. After standing for 15 min. the protein precipitate was centrifuged off. From the supernatants the non-protein component was precipitated by acetone with a trace of solid NaCl. The resulting precipitate was washed once with acetone and once with ether in the centrifuge, then dried in a vacuum desiccator.

This product dissolved rather easily in water, at first forming droplets which then gave clear solutions of pH 6·6–6·8. All protein reactions were negative; sulphosalicylic acid, however, produced a faint turbidity in the solutions. The ash content of the preparation was, on the average, 25%. The orcinol reaction was strongly positive. After acid hydrolysis adenine was isolated as picrate, m.p. 279°. (Found: N, 29·1. Calc. for $C_{11}H_{10}O_8N_8$: N, 29·3%.) The molecular proportions of Mg : total organic P : 7 min. P were found to be 1·99 : 3·0 : 2·0; inorganic P was absent. These results suggested the presence of a magnesium salt of adenosinetriphosphate (ATP). This was confirmed by the preparation of the acridine salt of ATP (Wagner Jauregg, 1936). For this purpose the deproteinization with salicylic acid was not suitable, since an acridine salt of salicylic acid, m.p. 132°, was found to contaminate the nucleotide precipitate. Therefore 2N-hydrochloric acid was used for deproteinization. The acridine precipitates were collected on a Büchner funnel, washed once with ethanol-ether (1 : 1), followed by ether. After one recrystallization from water they melted at 210° with decomposition. These acridine precipitates could be fractionated to yield one compound which was insoluble in cold water and only very sparingly soluble in ethanol, and a second compound which was much more soluble in both these solvents. More than 90% was present in the form of the insoluble compound which, after one recrystallization from water, gave a satisfactory analysis for the acridine salt of ATP. (Found: N, 10·8. Calc. for $C_{10}H_{16}O_{13}N_5P_3 + 2C_{13}H_9N$: N, 11·3%.) The ratio total organic P : 7 min. P was 1·5. The melting point remained unchanged at 210° (decomp.).

Preparation of the barium salt of adenosinetriphosphate

Finely powdered myotoxin (5 g.) was dissolved in a mortar with 100 ml. of cold water, added in small quantities. Any insoluble material was removed either by filtration (Whatman paper no. 54) or centrifugation. All further steps were carried out at temperatures near 0° with ice-cold reagents. The clear solution was brought to pH 4·5 by adding glacial acetic acid drop by drop with constant stirring. The precipitate formed was spun down after 15 min. and discarded. To the clear supernatant a 25% (w/v) solution of Ba acetate in water was added in slight excess. The pH rose to about 6. The resulting precipitate was washed with water and purified as the Hg salt (Lohmann, 1931). The final Ba precipitation was carried out at pH 4·5–4·6. This second Ba precipitate was washed twice with water, 50% (v/v) ethanol, absolute ethanol and ether.

The barium salt obtained corresponded to $Ba_{1.5}$ ATP. The water content of the preparation varied in different batches; 4, more often 6, H_2O were found in the samples. Differences in the barium content were slight and probably due to variations in pH during the final precipitation, as reported by Kiessling (1934) for the barium salt of inosinetriphosphate. (Found: N, 8·5; P, 11·4; Ba, 26·5. Calc. for $C_{10}H_{13}O_{13}N_5P_3Ba_{1.5} + 6H_2O$: N, 8·6; P, 11·4; Ba, 25·2%.) The average yield was 3 g.

The magnesium salt of adenosinetriphosphate

Solutions of the magnesium salt of ATP could easily be prepared by treating the barium salt with magnesium sulphate. Such solutions containing between 30 and 50 mg. of Mg ATP/ml. were viscous, frothed easily and resembled solutions of polymers such as nucleic acids rather than solutions of mononucleotides. Difficulties arose when attempts were made to isolate the magnesium salt. Precipitation from aqueous solutions with ethanol or acetone was incomplete and the solubilities of the preparations thus obtained varied from batch to batch. Under apparently identical conditions of isolation preparations were obtained which were easily soluble in water, whereas others dissolved only on prolonged shaking and some were scarcely soluble at all. The yields of Mg ATP varied between 60 and 93%; from the less readily soluble preparations solutions were obtained which contained from 15–25 mg./ml. at 18°. Attempts to obtain soluble magnesium salts using the method of Neuberg & Sabetay (1925) also failed to yield uniform preparations.

It was outside the scope of this work to investigate why pure preparations of Ba ATP yielded magnesium salts which differed so widely in their physical properties, but one observation seems worth mentioning. When Mg ATP preparations obtained by methanol precipitation were redissolved in water a highly viscous mass remained undissolved. This viscous residue was easily soluble in 0·05N-

hydrochloric acid. Reprecipitation with methanol yielded a colourless, amorphous substance. Aqueous solutions of this substance were slightly opaque, their N : P : 7 min. P ratio was 10·02 : 5 : 2·95, values suggestive of diadenosine pentaphosphate, the dinucleotide which Embden (1932) suggested was present in heart muscle.

One of the reasons for the failure to obtain reproducible preparations of Mg ATP became obvious when the extraordinary instability of this compound in aqueous solutions was noticed. In initially clear solutions a slight turbidity appeared when they were left at room temperature (18°) for several hours. This turbidity increased progressively and a precipitate formed. A similar observation has been reported by Barrenscheen & Filz (1932). Precipitates appeared at once when solutions of Mg ATP were boiled. In both cases the precipitates consisted of colourless, slightly curved needles of varying length.

It seemed of interest to study the changes occurring in solutions of Mg ATP.

Dephosphorylation of the magnesium salt of adenosinetriphosphate

A clear, neutral solution of 500 mg. of Mg ATP in 15 ml. of water was placed in a water bath which was slowly heated, the boiling point being reached within 30 min. When the temperature had reached 50° the solution became increasingly turbid, flocculation was noticed at about 60° and a precipitate started to settle out at about 65°. The mixture was kept for 1 hr. in the boiling-water bath under a reflux condenser and then cooled with running water. In a filtered sample no more precipitate was formed on further heating. The precipitate was collected on a Büchner funnel, washed with hot water and dried over calcium chloride. The substance was only slightly soluble in hot water. It dissolved in dilute mineral acid, but could not be reprecipitated on neutralization. It contained only traces of inorganic P, the orcinol reaction was positive and organic and acid-labile P were found. The ratios of N : P : 7 min. P were 4·83 : 3·0 : 1·93, suggestive of slightly impure ATP. The yield was on the average about 25 % of the Mg ATP originally present.

The filtrate from this product contained large amounts of inorganic phosphate. On addition of dilute ammonia $(NH_4)MgPO_4$ was precipitated which, after one recrystallization, contained 5·9 % N (calc. N, 5·7 %). The filtrate from this precipitate contained only a trace of inorganic phosphate, the orcinol reaction was strongly positive and organic phosphate was present which could be partly hydrolysed in 7 min. For the isolation of the substance responsible for these reactions the solution was acidified with glacial acetic acid, and lead acetate solution was added until no further precipitate formed. After several washings with water the precipitate was decomposed with hydrogen sulphide. In the clear filtrate acetone produced an oily precipitate, which, after collecting on the centrifuge, was hardened with acetone and dried with absolute ether. The product showed signs of crystallization and melted with decomposition at 95–100°. It was extremely hygroscopic, the aqueous solution giving a strongly acid reaction. On analysis it contained N : P : 7 min. P in the ratio 5·4 : 2 : 1·04. These values suggest the formation of adenosinediphosphate.

The fraction of Mg ATP which precipitated out during the heating could be dephosphorylated in the same way by redissolving it in acid, neutralizing and reheating.

The amount of insoluble Mg ATP was found to decrease to about 10 % when the heating was applied to solutions of pH 4·5. At this pH heating for periods longer than 1 hr., gave rise to the formation of lower split products (Table 1).

Preparation of barium adenosinediphosphate

For the preparation of Ba adenosinediphosphate (ADP) the following procedure was adopted. $Ba_{1·5}$ ATP was dissolved in 0·2 N-HCl, the Ba was removed with the calculated amount of $MgSO_4$ (10 % (w/v) in water). To the Ba-free

Table 1. *Dephosphorylation in magnesium adenosinetriphosphate* (Mg *ATP*) *solutions at* pH 4·5

(Mg ATP solution prepared at 0°: $Ba_{1·5}$ ATP dissolved in 0·2 N-HCl, Ba removed with calculated amount of $MgSO_4$, N-NaOH added to pH 4·5. (1·088 mg. total P/ml., trace of inorganic P, N : 7 min. P = 5 : 1·94.) Samples (in individual graduated tubes) were placed simultaneously in a water bath at 20°. After having reached this temperature the first sample was removed, followed by others at specified times and temperatures. All were immersed in ice water, made up to volume and filtered.)

Time (min.)	Bath temp. (°)	Appearance of sample	(mg./ml. of filtrate) Total P	Inorganic P	Organic P	7 min. P	N	Ratio N : 7 min. P
0	20	Clear	1·09	0·05	1·04	0·65	0·82	5 : 1·80
18	92	Turbid	1·08	0·06	1·02	0·63	0·80	5 : 1·78
20	100	Precipitate	0·96	0·06	0·90	0·60	0·77	5 : 1·75
30	100	Precipitate	0·91	0·19	0·73	0·38	0·73	5 : 1·17
60	100	Precipitate	0·89	0·28	0·61	0·28	0·73	5 : 0·88
90	100	Precipitate	0·89	0·34	0·55	0·23	0·74	5 : 0·69
120	100	Precipitate	0·90	0·40	0·49	0·17	0·74	5 : 0·50

solution N-NaOH was added to bring the pH to 4·5. Of this solution 100 ml. were heated for 60 min. in a boiling-water bath, cooled and the precipitate filtered off. Table 2 shows the results of P and N estimations in this filtrate and also the values in the solution before heating was started. The filtrate was cooled in ice, acidified with 0·2N-HNO_3 and Lohmann reagent added in slight excess. The resulting precipitate, after washing, was resuspended in water, decomposed by H_2S, the filtrate from HgS aerated to remove excess H_2S. A saturated solution of Ba acetate in 50% (v/v) ethanol was added and finally absolute ethanol to bring its total concentration in the solution to 50%. The resulting Ba precipitate was washed with 50% ethanol, ethanol and absolute ether and finally dried in a vacuum desiccator.

Table 2. *Dephosphorylation in a magnesium adenosinetriphosphate (Mg ATP) solution used for the preparation of adenosinediphosphate (ADP)*

(Mg ATP solution prepared as in Table 1. Samples were taken before and after heating in a boiling-water bath for 60 min.)

	In solution at start	In filtrate after heating
Total P (mg./ml.)	0·93	0·81
Inorganic P (mg./ml.)	0·04	0·24
Organic P (mg./ml.)	0·89	0·57
7 min. P (mg./ml.)	0·59	0·27
N (mg./ml.)	0·68	0·66
Organic P/7 min. P	1·51	2·09
Ratio N : P	5·09 : 3	5·13 : 2

The Ba ADP retained $3H_2O$ on drying *in vacuo* at 50°. (Found: N, 10·3; P, 8·9; 7 min. P, 4·5. Calc. for $C_{10}H_{12}O_{10}N_5P_2Ba_{1·5}+3H_2O$: N, 10·2; P, 9·1; 7 min. P, 4·5%.) (Found, after drying over P_2O_5 at 113°: N, 11·1. Calc.: N, 11·1%.) Yield 80%.

To establish the identity of the chemically prepared Ba ADP with preparations obtained in the usual way by enzymic dephosphorylation of ATP some derivatives were prepared. The acridine salt, after one recrystallization from water had the correct m.p. 215° (Wagner Jauregg, 1936). (Found: N, 13·6. Calc. for $C_{10}H_{15}O_{10}N_5P_2+C_{13}H_9N$: N, 13·9%.) The barium salt was regenerated from the acridine salt. (Found, after drying at 50° *in vacuo*: C, 17·7; H, 2·5; N, 10·1; Ba, 29·3. Calc. for $C_{10}H_{12}O_{10}N_5P_2Ba_{1·5}+3H_2O$: C, 17·5; H, 2·7; N, 10·2; Ba, 30·1%.)

Adenine picrate was isolated from Ba ADP solution after hydrolysis in N-hydrochloric acid. (Found: N, 29·5. Calc. for $C_{11}H_{10}O_8N_8$: N, 29·3%.) Adenylic acid, prepared from Ba ADP according to Lohmann (1932) and isolated using the acetone method (Embden & Zimmermann, 1927) had m.p. 199°. The mixed m.p. with samples of adenylic acid prepared from muscle and from Ba ATP was identical. The Klimek & Parnas (1932) modification of the Böeseken reaction indicated the presence of a 5-nucleotide.

The ADP obtained by chemical means reacted with N-acid and with nitrite as would be expected, ADP having the constitution postulated by Lohmann (1935) and confirmed by Gulland & Farrell Walsh (1945).

The formation of inorganic phosphate when ATP solutions were heated to 100° could also be observed with salts other than the magnesium salt, e.g. the sodium and barium salts, but these salts were less suitable for the preparation of Ba ADP. Isolation of the pure compound was difficult in one case due to the solubility of the fraction of Na ATP which remained unsplit, and in the other due to the very low solubility of $Ba_{1·5}$ ATP at pH 4·5. The increase of inorganic phosphate in solutions of Na ATP and Ba ATP when heated to 100° for 60 min. is shown in Table 3.

Table 3. *Dephosphorylation in sodium and barium adenosinetriphosphate solutions at* 100°

(Ba ATP solution: saturated solution of $Ba_{1·5}$ ATP at 18°. Na ATP solution: prepared from $Ba_{1·5}$ ATP as in Tables 1 and 2.)

	Na ATP	Ba ATP
Total P (mg./ml.)	2·14	0·56
Inorganic P at start (20°) (mg./ml.)	0·11	0·01
Inorganic P after 60 min. in boiling-water bath (mg./ml.)	0·43	0·16

Inosinediphosphoric acid could be isolated by the same method from solutions of magnesium inosinetriphosphate, prepared from $Ba_{1·5}$ ATP according to Kleinzeller (1942).

Evidence for the presence of inosinetriphosphate in myotoxin preparations

When myotoxin solutions, after deproteinization with 2N-HCl, were treated with acridine, a precipitate was obtained, of which 90% consisted of the acridine salt of ATP, as already mentioned. The amounts of the ethanol-soluble acridine compound available were so small that purification by repeated recrystallization was not attempted. When this acridine compound was split by treating with sodium carbonate, the acridine filtered off by suction and the last trace removed by ether extraction, the ratio of total organic P : 7 min. P in the filtrate was 3 : 2 and on hydrolysis it yielded hypoxanthine which was isolated as the picrate. (Found: N, 25·8. Calc. for $C_{11}H_9O_9N_7$: N, 25·6%.) An acridine salt of inosinetriphosphoric acid, prepared from ATP, showed the same solubilities in water and ethanol as the ethanol-soluble fraction obtained from myotoxin.

Myotoxin as a source of adenosinetriphosphate

In order to test whether extraction of acetone-dried muscle with saline was a satisfactory method for the preparation of ATP the following experi-

ment was carried out. Saline and trichloroacetic acid extracts were simultaneously prepared from acetone-dried rabbit muscle. Pentose : 7 min. P ratios of 1 : 2·02 and 1 : 2·00, respectively, were found. The inorganic P content was 0·252 mg./ml. in the saline and 0·376 in the trichloroacetic extract. After leaving both extracts for 24 hr. at 15°, 0·243 and 0·405 mg. of inorganic P were found, figures which rule out enzymic activity in the saline extract. However, when the saline extract was heated slowly and then boiled with a total exposure to heat of 15–20 min. as in the preparation of myotoxin, the inorganic P rose to 0·286 mg./ml. of filtrate and a pentose : 7 min. P ratio of 1 : 1·53 was found, indicating a loss of ATP.

DISCUSSION

The work described in this paper formed part of an investigation on traumatic shock carried out under the aegis of the Medical Research Council during the years 1942–5, a full report of which has been deposited at the Library of the National Institute for Medical Research, London, N.W. 3.

The isolation of ATP from myotoxin led to the elaboration of a simple method for the preparation of pure ATP, applicable to small or large amounts of acetone-dried muscle. This material seems to be a good source of ATP. The inactivation of adenosinetriphosphatase by acetone, described by Barrenscheen & Lang (1932), may well explain the stability of ATP in such muscle preparations.

Traces of inosinetriphosphate were found to be present in some batches of ATP prepared by the method described. It seems likely that this compound was preformed in the muscle before immersion in acetone. Lohmann (1932) has shown that under certain conditions inosinetriphosphate can be formed in frog muscle by deamination prior to dephosphorylation.

A study of the magnesium salt of ATP led to some interesting observations. Evidence has been obtained of the presence of a polynucleotide precipitated by methanol from Mg ATP solutions. A preparation of the same composition was isolated by Embden (1932) from heart muscle and later confirmed by Ostern (1934).

Dephosphorylation in Mg ATP solutions occurs at room temperature and is accelerated by elevated temperatures. Suitable conditions have been found under which this breakdown of Mg ATP can be utilized for the preparation of adenosinediphosphate.

On the basis of the observations mentioned it seems doubtful whether Dyckerhoff's (1939) method of deproteinization as given for myotoxin is the best procedure for the preparation of ATP. The main constituent of myotoxin is Mg ATP which is easily dephosphorylated when its solutions are exposed to heat. The yield of ATP from saline extracts could probably be improved by a method of deproteinization avoiding high temperatures.

SUMMARY

1. A toxic principle present in the myotoxin of Dyckerhoff *et al.* (1939) has been identified as magnesium adenosinetriphosphate.

2. Traces of inosinetriphosphate have also been found in some batches of myotoxin.

3. A chemical method for the preparation of adenosinediphosphate (as barium salt) from magnesium adenosinetriphosphate is described.

I wish to thank Prof. H. N. Green for his interest in this work. My thanks are also due to Prof. H. A. Krebs, F.R.S., for a sample of adenosinetriphosphate and for reading the proofs of this paper.

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The Relationship between the Constitution and the Effect of Chemical Compounds on Plant Growth

2. QUATERNARY AMMONIUM SALTS

By R. L. JONES, J. MADINAVEITIA, T. P. METCALFE and W. A. SEXTON
Imperial Chemical Industries Limited, Dyestuffs Division, Research Laboratories, Blackley, Manchester

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It has been recorded by Jones, Metcalfe & Sexton (1949) that certain quaternary ammonium salts containing the substituted phenoxyethyl radical exert a differential effect upon the germination of oat and rape seeds and it was suspected that the activity of some of them might be dependent upon their quaternary salt function rather than the presence of substituted phenoxyethyl radicals. Some quaternary ammonium salts are known to react readily with compounds containing thiol groups, and in this respect they resemble $\alpha\beta$-unsaturated ketones and lactones. The bactericidal activity of these ketones and lactones has been attributed to their ability to react with thiols (Geiger & Conn, 1945; Cavallito & Haskell, 1945). Since $\alpha\beta$-unsaturated lactones are also active in suppressing the germination of seeds (see, for example, Veldstra & Havinga, 1943, 1945) it was considered possible that quaternary ammonium salts which react with thiols, for example, those containing benzyl radicals, might show herbicidal activity. Support for this hypothetical basis for biological activity in quaternary salts containing benzyl radicals was obtained in preliminary experiments which showed that the thiol-dependent enzymes, urease and glyoxalase, were inhibited by several of them. Evidence for the importance of thiol enzymes in plant growth has recently been provided by Thimann & Bonner (1949).

METHODS

Preparation of compounds

The compounds were obtained by the conventional method, by reaction of alkyl or benzyl halides or dimethyl sulphate with a tertiary base (with or without a solvent). Methosulphates were converted to iodides by the addition of NaI or KI to their aqueous solutions. Yields were generally good. The compounds which are new are listed in Table 1, together with their physical characteristics and analytical data. A typical preparation, that of methyldiethylbenzylammonium iodide, is described below.

Methyldiethylbenzylammonium iodide. (i) *Direct method.* Methyl iodide (2 ml., 3·5 mol.) was added to a solution of diethylbenzylamine (1·46 g., 1 mol.) in methanol (10 ml.). After warming for 15 min. the cooled reaction mixture was treated with ether (35 ml.) and the crystalline precipitate separated, washed with ether and dried. Yield 1·5 g. (55%), m.p. 147–149°. Recrystallization from ethanol or water afforded material, m.p. 149–151°. Alternatively, the base was warmed with methyl iodide without a solvent and the resulting solid purified by recrystallization. (ii) *Through the methosulphate.* Diethylbenzylamine (1·08 g., 1 mol.) and methyl sulphate (2 ml., 3·2 mol.) were dissolved in methanol (10 ml.) and warmed for 0·5 hr. After cooling and addition of ether (40 ml.) the lower oily layer of quaternary salt was washed with ether, dissolved in water (10 ml.) and, after clarification with charcoal, treated with excess of KI. The precipitated quaternary iodide (1·3 g., 64%) was separated and purified, m.p. 149–151°, by recrystallization from water.

Biological methods

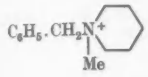
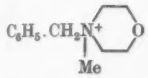
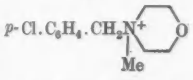
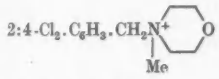
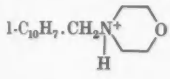
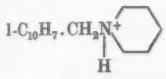
The biological test methods, using germinating oat and rape seeds on agar in test tubes, are described by Jones *et al.* (1949). In the tables which follow, two plus signs indicate the highest measure of activity (at least 50% inhibition of root length in more than half the seeds). Control seeds were grown on agar.

RESULTS

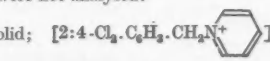
The results of routine tests are recorded for comparative purposes in Tables 2–4. Table 2 indicates the effect of varying molecular weight upon the activities of quaternary ammonium salts, using variation of alkyl chain length (the alkyl groups being of the normal series except where otherwise indicated) and using aromatic groups in certain instances.

Attention was next directed (Table 3) towards benzyl compounds, as it was desirable to know whether the benzene ring substituents which are known to increase activity in the phenoxyacetic acid series had similar effects in the quaternary benzyl ammonium salts. We studied the effect of introduction of chlorine or nitro in the aromatic ring and the effect of the fusion of a second ring (giving 1-naphthyl) on the biological activity of quaternary derivatives of trimethylammonium, methyldiethylammonium, morpholinium and piperidinium ions. In each group of compounds the anion was identical.

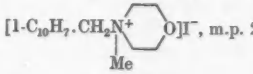
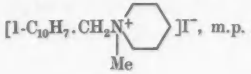
Table 1. *New compounds prepared*

Compound			Analysis	
Cation	Anion	Melting point	Found	Required
$C_6H_5.CH_2N^+MeEt_2$	I^-	149–151°	C, 46·9 H, 6·9	47·2 6·6
	I^-	126°	I, 38·9	40·0
	I^-	152–156°	I, 40·1	39·9
	I^-	171°	N, 4·3	4·0
$2:4\text{-}Cl_2.C_6H_3.CH_2N^+Me_3$	I^-	208° (sinters 192°)	C, 34·3 H, 4·3	34·7 4·1
$2:4\text{-}Cl_2.C_6H_3.CH_2N^+MeEt_2$	I^-	165° (sinters 148°)	C, 38·7 H, 4·9	38·6 4·5
	I^-	88–90° (with 1 mol. H_2O)	C, 33·5 H, 7·9	33·5 8·1
$1\text{-}C_{10}H_7.CH_2N^+Et_2H$	Cl^-	214°	C, 71·3 H, 7·9	75·8 8·1
	Cl^-	233°	C, 68·2 H, 6·7	68·5 6·8
	Cl^-	229°	C, 72·7 H, 7·5	73·2 7·7

The following new compounds were also made, but were not analysed:

$[p\text{-}Cl.C_6H_4.CH_2N^+Me_3]Cl^-$, hygroscopic solid;  , m.p. 102–103°;

$[1\text{-}C_{10}H_7.CH_2N^+MeEt_2]I^-$, could not be crystallized;

 , m.p. 200°;  , m.p. 206°.

In addition to the above compounds, the following sulphonic acid derivatives were found to be completely inactive at 100 p.p.m.

$C_6H_5CH_2N^+Me_2C_6H_4SO_3^-$ (*m* and *p*)
$O_3^-SC_6H_4CH_2N^+Me_2C_6H_5$ (*m* and *p*)
$C_6H_5CH_2N^+Me_2CH_2C_6H_4SO_3^-$ (*m* and *p*)

Attention was finally directed to the effect of variation of substituents on the nitrogen atom of quaternary benzylammonium or substituted benzylammonium ions (Table 4). There was some anion variation dictated by the immediate availability of compounds for test, but sufficient valid comparisons were available to permit an assessment of the effect of structural changes in the cation.

DISCUSSION

Marked herbicidal activity in quaternary salts containing *n*-alkyl chains does not appear until the decyl compound. Higher in the series the activity falls off, at least against rape (Table 2). This is a typical homologous series effect in which the variation in biological activity is largely determined by the physico-chemical properties of the molecule. The highest herbicidal activity occurs when the compounds have marked surface activity as judged by the foaming of aqueous solutions, and in this the bactericidal and fungicidal properties of the same compounds are recalled. There is clearly a maximum activity against rape between C_{10} and

C_{14} though its position has not been determined with precision. The position of the maximum against oats is not revealed by the results, but it would appear that it is probably at a somewhat greater chain length than in the case of rape. This difference results in the appearance of selective action in the case of the octadecyl compound, which has passed the peak with rape, but is still very active against oats. It is to be noted that molecular weight, as such, is not a factor determining activity. The last three compounds in Table 2, containing naphthyl radicals, are of low activity compared with *n*-decyl- and *n*-dodecyl-trimethylammonium halides, which are of comparable molecular weight.

In Table 3, it is shown that the introduction of chlorine into the benzene ring or substitution of phenyl by α-naphthyl does not cause the enhancement of activity characteristic of the substituted phenoxyacetic acids. Nor does the *p*-nitro group have a marked effect. A further difference from the phenoxyacetic acid series is seen in the sensitivity of oats to the benzyl substituted quaternary salts and the insensitivity of rape. It is interesting to note that, in the groups of compounds recorded in Table 3, activity is but slightly influenced, if at all, by substituents in the benzyl radical, whereas it is very considerably influenced by the other substituents on the nitrogen atom. Thus very little activity is shown by quaternary salts derived from *N*-methylmorpholine or ethyldimethylamine, but considerable activity in those derived from tri-

Table 2. *Activities of homologous quaternary ammonium salts on the germination of rape and oats*

(Activities in this and subsequent tables shown by + and − signs as stated in text (p. 110).)

Compound $[Me_3N^+R]X^-$		Concentration	Activity	
R	X	(p.p.m.)	Rape	Oats
CH_3	I	100	−	+
C_2H_5	I	50	−	+
C_3H_7	Br	100	−	−
C_4H_9	Br	50	−	−
C_6H_{13}	Br	50	−	−
C_7H_{15}	Br	50	−	−
C_8H_{17}	Br	50	−	+
$C_{10}H_{21}$	Br	50	++	++
		10	++	+
		1	−	.
$C_{12}H_{25}$	Br	50	++	++
		10	++	++
$C_{14}H_{29}$	Br	50	++	++
		10	++	++
$C_{16}H_{33}$	Br	50	+	++
		10	.	++
$C_{18}H_{37}$	Cl	50	+	++
C_3H_5 (allyl)	I	50	−	+
C_6H_5	I	50	−	++
		10	.	+
$C_6H_5CH_2$	Cl	50	−	+
compound $[1\text{-}C_{10}H_7CH_2N^+Me_2R]Cl^-$				
R				
CH_3		100	−	++
		50	.	+
C_2H_5		100	−	+
C_3H_7		100	+	+

Table 3. *Effect of variation of substituents in the benzyl radical of quaternary ammonium salts on the activities against germinating rape and oats*

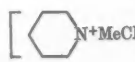
Compound		Concentration	Activity	
Type	Radical R	(p.p.m.)	Rape	Oats
$[Me_3N^+CH_2R]Cl^-$	C_6H_5	50	−	+
	o-ClC_6H_4	50	−	+
	2:4-$Cl_2C_6H_3$	100	+	++
		50	.	++
		10	.	+
	p-$NO_2C_6H_4$	50	−	+
	1-$C_{10}H_7$	100	−	++
		50	.	+
$[EtMe_2N^+CH_2R]I^-$	C_6H_5	50	−	+
	2:4-$Cl_2C_6H_3$	100	−	+
	1-$C_{10}H_7$	100	−	+
	C_6H_5	100	−	+
	o-ClC_6H_4	100	−	+
	2:4-$Cl_2C_6H_3$	100	−	+
	p-$NO_2C_6H_4$	100	−	+
	1-$C_{10}H_7$	100	−	+
	C_6H_5	100	−	++
		50	.	++
	o-ClC_6H_4	50	−	++
	1-$C_{10}H_7$	50	−	++
		10	.	+

Table 4. *Effect of variation of the* N*-substituents on the activities of quaternary ammonium salts containing benzyl and substituted benzyl radicals against germinating rape and oats*

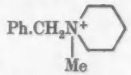
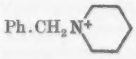
Compounds					
Type	Radicals R	Anion X	Concentration (p.p.m.)	Rape	Oats
$[C_6H_5CH_2N^+R_3]X^-$	Me_3	Cl^-	50	–	+
	Et_3	I^-	50	–	++
			10	.	–
	$Me.Et_2$	I^-	50	–	+
	$Me_2.Ph$	I^-, Cl^-, Br^- and SO_4Me^-	50	–	++
		Cl^-	10	–	+
	Me.Et.Ph	I^-	100	–	++
			50	.	–
	$Me.C_5H_{10}$ (piperidine)	I^-	50	–	+
	$Me.C_4H_8O$ (morpholine)	I^-	100	–	+
	C_5H_5 (pyridine)	Cl^-	100	–	+
$[2:4\text{-}Cl_2C_6H_3CH_2N^+R_3]I^-$	Me_3		100	+	++
			50	.	++
			10	.	+
	$Me.Et_2$		100	–	+
	$Me.C_4H_8O$ (morpholine)		100	–	+
	C_5H_5 (pyridine)		50	–	++
			10	.	+
$[1\text{-}C_{10}H_7CH_2N^+R_3]I^-$	Me_3		100	–	++
			50	.	+
	$Me.Et_2$		100	–	+
	$Me.C_4H_8O$ (morpholine)		100	–	+
	$Me.C_5H_{10}$ (piperidine)		100	+	++
			50	.	++
	C_5H_5 (pyridine)		50	–	++
			10	.	+

methylamine and *N*-methylpiperidine. From the selective action and from the absence of surface-active properties in this group of compounds, it is to be concluded that their mode of action is different from that of the normal alkyl compounds recorded in Table 2. In spite of this, the physical properties of the quaternary benzyl compounds are not without significance, as is shown by the complete loss of activity on introduction into the molecule of a sulphonic acid group.

The realization of the decisive part played by the other substituents on the nitrogen atom of quaternary benzylammonium salts prompted further study. The results given in Table 4 provide additional information on the effect of substituents and include also a comparison of the effects of different anions with the same cation. At the concentrations examined these showed no differences. It will be seen that from the point of view of achieving the highest degree of biological activity, pyridine was a much better base than *N*-methylmorpholine or methyldiethylamine in the 2:4-dichlorobenzyl series, but this was not so in the unsubstituted benzyl series. Again, trimethylamine is a favourable base in the 2:4-dichloro series, but not in the 1-naphthyl series.

Table 5. *Comparison of herbicidal activities with the alkylating activities of quaternary benzyl cations*

(Herbicidal activities measured against oats at 50 p.p.m.)

Cation	Herbicidal activity	Alkylating activity
$PhCH_2N^+Et_3$	++	Slightly reactive
$PhCH_2N^+Me_3$	+	Slighdy reactive
	+	Slightly reactive
$PhCH_2N^+Me_2Ph$	++	Reactive
	+ (at 100 p.p.m.)	Reactive
$Ph.CH_2N^+Me_3$	++	Not reactive

It is of interest to compare the herbicidal activities of the benzyl quaternary salts with their alkylating (benzylating) ability. Although accurate quantitative data on the chemical side are lacking,

the work of Snyder & Speck (1939) and Snyder, Smith & Stewart (1944) on the benzylation of thiol compounds, acetoacetate and malonate provides useful information. From this and the data in Tables 1 and 4, the comparisons given in Table 5 have been made. Thus it can be seen that chemical affinity for a reactive hydrogen does not appear to run parallel with biological activity.

As has been suggested in the case of contact insecticides (Davies & Sexton, 1948), it is probable that biological activity depends upon having the correct degree of chemical reactivity in the group concerned, balanced with physical properties which ensure maximum ease of penetration to the site of action.

After our work had been completed, a paper by Mitchell, Wirwille & Weil (1949) appeared, recording plant-growth regulatory activity in certain quaternary salts containing benzyl and substituted benzyl radicals. Prill, Barton & Solt (1949) have also observed a reduction of the growth of wheat roots by various surface-active compounds of which the most active were two quaternary ammonium salt derivatives, both of which reduced root growth by 50% at concentrations of 5 p.p.m.

SUMMARY

1. The herbicidal activity of quaternary ammonium salts has been examined by a laboratory seed-germination technique, using rape and oats.

2. In the homologous series of *n*-alkyltrimethyl ammonium salts activity against both species rose as the series was ascended. In the case of rape, activity diminished after C_{14}. In the case of oats the position of the maximum was not determined, but it appeared to be higher.

3. In a series of quaternary salts containing benzyl or substituted benzyl radicals, oat seeds were more susceptible than rape.

4. In contrast to the results in the phenoxyacetic acid series, the herbicidal activity was but little affected by the introduction of substituent groups (e.g. chlorine) into the benzene ring. A sulphonic acid group brought about complete inactivation.

5. The activity was greatly influenced by the nature of the other substituent groups on the nitrogen atom.

6. The results are discussed in relation to the known ability of certain quaternary salts to alkylate thiol groups.

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Fluorescence of Riboflavin and Flavin-adenine Dinucleotide

By G. WEBER (Beit Memorial Fellow)

Biochemical Laboratory, University of Cambridge

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The fluorescence of riboflavin has been extensively studied with a view to the determination of this substance. Relatively little work has been done on the variations of this property in the compounds of riboflavin of biological importance such as flavin-adeninedinucleotide (FAD) and the flavoproteins.

The variations in fluorescence under different conditions, and notably after addition of quenchers, can give some indication of the configuration of a molecule. The following is a study of the fluorescence of riboflavin and FAD from this point of view.

EXPERIMENTAL

The intensity and the degree of polarization of the fluorescent light and the absorption spectrum were determined with solutions of riboflavin and FAD under various conditions.

For the measurement of the intensity of the fluorescence, the exciting light was filtered through a combination of blue and green filters (Corning glass 5850 and 978), while the fluorescent light reached the photocell through an orange filter (Corning glass 351). The photocell was of the barrier-layer selenium type. The same combination of filters was used in the observations of the polarization of the fluorescence. The exciting light was linearly polarized.

The percentage of polarization of the fluorescent light is given by the identity (Wood, 1934)

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}},$$

where $I_{\parallel}$ is the intensity of the polarized wave with its electric vector parallel to that of the exciting light, while $I_{\perp}$ is the intensity of the polarized wave with its electric vector normal to that of the exciting light. The measurement of the polarization of the fluorescence was made by means of a Savart plate and an Arago compensator. The latter was calibrated by viewing the light reflected from the face of a glass prism, the fraction of polarized light being calculated by the use of the Fresnel equations (Gaviola & Pringsheim, 1926). For the measurements of low degrees of polarization a pile of glass plates was placed between the source and the compensator (Perrin, 1936) so as to increase the amount of polarized light in the latter, and a new calibration curve was made. The general arrangement of the apparatus was similar to that described by Perrin (1929) and will be described in detail in another paper.

The absorption spectrum of the solutions were measured with a Beckman photoelectric spectrophotometer. The viscosity of the glycerol-water mixtures used in some experiments was obtained with an Ostwald viscometer.

RESULTS

Fluorescence of riboflavin and the action of quenchers

The fluorescence of flavin-adeninedinucleotide (FAD) was found to be much weaker than that of free riboflavin and this was traced, as later shown, to a quenching effect of the adenine part of the molecule. A similar quenching effect is produced by the addition of adenine compounds or of purines to solutions of riboflavin. The quenching effect of purines differs considerably from that of electrolytes like potassium iodide or potassium thiocyanate (Weber, 1948). The fluorescence of riboflavin is quenched by a number of substances: electrolytes, diphenols, purines and pyrimidines. Table 1 shows the relative efficiency of these quenchers. I/I_0 is the ratio of the fluorescence intensities after and before the addition of the quencher respectively. It is also the ratio of the corresponding fluorescence efficiencies, provided no change in the absorption spectrum of the fluorescent solution over the range of absorbed and emitted wavelengths takes place on addition of quencher (Sveshnikoff, 1936). Table 1 also shows that silver ions are the most effective of all quenchers studied. They are followed by the purines, which exhibit small differences among themselves. Breaking of the pyrimidine ring is followed by complete loss of the quenching ability as shown by allantoin. Potassium iodide, the most effective of the electrolytes is slightly less effective than the diphenols.

Table 1. *Quenching of the fluorescence of riboflavin by different substances*

(I/I_0 is the relative fluorescent efficiency of an aqueous solution containing 10^{-2} M-quencher and $6{\cdot}9 \times 10^{-6}$ M-riboflavin. Measurements made at room temperature. pH values varied from 6 to 8.)

Quencher	I/I_0
Adenine	0·50
Adenosine	0·50
Hypoxanthine	0·51
Caffeine	0·53
Muscle adenylic acid	0·63
Thymine	0·71
Cytosine	0·77
Allantoin	0·99
Potassium iodide	0·72
Hydroquinone	0·66
Catechol	0·65
Silver nitrate	0·03
Silver acetate	0·03

Action of potassium iodide. The quenching of the fluorescence of dyes in solution has been explained as due to collisions of the second kind, i.e. collisions

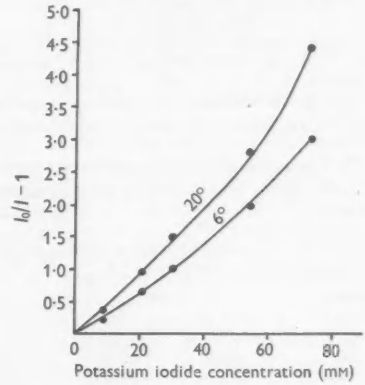

Fig. 1. Quenching of the fluorescence of riboflavin by KI. Riboflavin concentration, 7×10^{-6} M; I_0/I, ratio of the intensities of the fluorescence before and after adding the quencher.

in which the electronic excitation energy is transformed into kinetic energy, as opposed to collisions of the first kind, in which the reverse process takes place. In this type of quenching the efficiency of the quencher at low concentrations is constant and proportional to the lifetime of the excited state of the fluorescence, while at higher concentrations the efficiency of the quencher increases steadily. This is apparent from Fig. 1, which depicts the quenching

of the fluorescence of a solution of riboflavin by potassium iodide at two different temperatures. From the slopes of the linear part of the curves it is possible to calculate the mean life of the excited state using an equation derived by Wawilov (1928). In this way it is found that the lifetime of the excited state of riboflavin is approximately 1×10^{-8} sec. On comparing the two curves shown in Fig. 1, it is clear that the quenching is more effective at the higher temperature owing to the increase in the number of collisions and the decrease in the viscosity of the solvent (Wawilov, 1928).

The quenching by collisions of the second kind results in a shortening of the lifetime of the excited state. In this case, according to Perrin (1929),

$$\frac{I_0}{I}=\frac{\tau_0}{\tau}, \qquad (1)$$

where τ_0 and τ are the mean values of the lifetime of the excited state before and after quenching. The ratio τ_0/τ can easily be deduced from measurements of the polarization of the fluorescence (Sveshnikoff, 1936). If p_i is the initial polarization of the fluorescence before quenching, p_o the fundamental polarization* and p the observed polarization after addition of quencher, we have

$$\frac{\tau_0}{\tau}=\frac{\frac{1}{p_i}-\frac{1}{p_o}}{\frac{1}{p}-\frac{1}{p_o}}. \qquad (2)$$

Table 2 gives the values of I_0/I and τ_0/τ, the latter calculated from equation 2, for a series of solutions of

Table 2. *Effect of quenching by potassium iodide on the lifetime of the excited state of the fluorescence of riboflavin*

(Glycerol-water mixture of viscosity 0·040 c.g.s. unit at 17°. Riboflavin concentration 7×10^{-6} M.)

KI concn. (M)	I_0/I	p	τ_0/τ
0	1	0·038	1
0·050	1·46	0·053	1·43
0·110	2·18	0·076	2·17
0·191	3·03	0·098	2·95
0·335	5·0	0·135	4·35
0·620	10·0	0·200	7·7

riboflavin in a glycerol-water mixture of viscosity 0·040 c.g.s. units at 17°, containing increasing amounts of potassium iodide. It was necessary to use glycerol-water mixtures as solvent in these experiments, in order to have an initial polarization which could be measured with some accuracy.

The agreement of the values quoted in Table 2 with Perrin's rule (equation 1) can be considered good except at high values of the ratio I_0/I. For an interpretation of this see Wawilov & Franck (1931) and Weber (1948).

Action of purines. The results obtained for the quenching by purines, stand in contrast to those just discussed for the quenching by potassium iodide. From several facts to be reported presently we have concluded that a non-fluorescent complex is formed between the riboflavin and the purine molecules.

Let $[F_o]$ denote the total concentration of riboflavin, $[C_o]$ that of quencher, and $[FQ]$ the concentration of complex the degree of dissociation of which is denoted by α. Then

$[F]$ = Free riboflavin concentration $= \alpha[F_o]$,
$[FQ] = (1-\alpha)\,[F_o]$,
$[Q]$ = Free quencher $= [C_o] - (1-\alpha)\,[F_o]$.

Since $[F_o]$ is negligibly small compared with $[C_o]$ we can write $[Q]=[C_o]$. Applying the law of mass action, we have

$$K=\frac{[F]\,[Q]}{[FQ]}=\frac{\alpha}{1-\alpha}\,[C_o]. \qquad (3)$$

If only the free molecules can emit, $I/I_0=\alpha$, and

$$\frac{[C_o]}{K}=\frac{I_0}{I}-1. \qquad (4)$$

If the concentration of quencher is plotted against $(I_0/I)-1$ the reciprocal of the slope gives the dissociation constant of the complex. The measurements show (Fig. 2) that, in contradistinction to the quenching by potassium iodide, K is a true constant

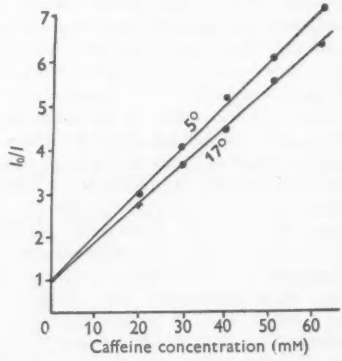

Fig. 2. Quenching of the fluorescence of riboflavin by caffeine. Riboflavin concentration, 7×10^{-6} M; I_0/I, ratio of the fluorescent intensities before and after addition of quencher.

* The so-called fundamental polarization, p_o, is the polarization attained in a medium where the fluorescent molecules cannot rotate appreciably in the interval between the absorption and the emission of light. In practice p_o differs little from the polarization in a medium of high viscosity, such as glycerol. For riboflavin p_o, as shown later, reaches approximately the theoretical maximum of 0·50.

for the whole range of concentrations studied. On the other hand, measurements of K at two temperatures reveal an increase of the dissociation of the non-fluorescent complex when the temperature is raised. Table 3 gives the dissociation constants at

Table 3. *Dissociation constants of purine-riboflavin complexes*

(Solvent 0·02 M-phosphate buffer pH 7·5.)

Quencher	Temp. (°)	$K \times 10^3$	S.E.
Adenosine	5	7·4	0·4
Adenosine	17	8·3	0·3
Caffeine	5	9·8	0·5
Caffeine	17	11·0	0·3

two temperatures for the quenching of the fluorescence of riboflavin by adenine and by caffeine at neutral pH. The variation of the quenching with temperature proved to be a perfectly reversible phenomenon. The figures of Table 3 show that the ratio $K_{5°}/K_{17°}$ is approximately the same for both purines and equal to 1·13. Assuming that the constants are determined with an accuracy of $\pm 5\%$, the van't Hoff isochore gives the heat of dissociation of the complex as $-1{\cdot}6 \pm 0{\cdot}6$ kg.cal./mol.

Effect of purine quenching on the lifetime of the excited state. When a solution of riboflavin is quenched by caffeine or adenosine, in contradistinction to potassium iodide, no increase in the polarization of the fluorescence took place, up to the highest ratio of I_0/I experimentally attained (about 20). Quenching by these substances has therefore no effect on the lifetime of the excited state of the fluorescence. In the event of complex formation (Weber, 1948)

$$\frac{\tau_0}{\tau} = \frac{I_0}{I}\,\alpha$$

and

$$\frac{\Sigma}{\tau_0} = \frac{\left(\frac{I_0\tau}{I\tau_0} - 1\right)}{\left(\frac{\tau_0}{\tau} - 1\right)}, \qquad (5)$$

where Σ is the mean life of the complex. Experimentally, as we have just mentioned, $\tau_0/\tau = 1$ for all accessible concentrations of quencher, and therefore $I/I_0 = \alpha$ which justifies the introduction of this expression in deriving equation 4. Equation 5 allows a calculation of Σ/τ_0 to be made from the experimentally determined values of I/I_0 and τ/τ_0. In the quenching of the fluorescence of riboflavin by purines it is only possible to get a minimum value for Σ/τ_0 as the change in the lifetime of the excited state on quenching falls within the experimental error of its determination, and it may be concluded that

$$\frac{\Sigma}{\tau_0} > 100 \quad \text{or} \quad \Sigma > 10^{-6}\ \text{sec.}$$

Modification of the absorption spectrum of riboflavin on addition of purines. Fig. 3 shows the absorption spectra of (1) a riboflavin solution (dark circles), (2) the same containing 0·050 M-adenosine (open circles), and (3) the same containing 0·052 M-caffeine (crosses). In the last two solutions the value of I_0/I was very approximately the same, namely 4·5. The modifications of the absorption spectrum consist of a decrease in the height of the two absorption maxima at 375 and 454 mμ. and in a displacement of the red edge of the absorption band towards longer wavelengths. The absorption spectrum of riboflavin in the presence of purines strikingly resembles the spectrum of FAD described by Warburg & Christian (1938).

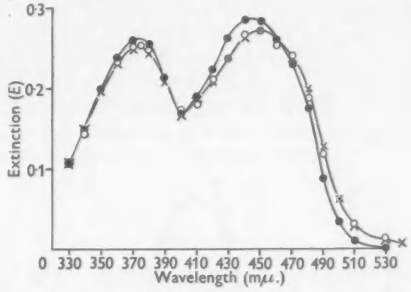

Fig. 3. Absorption spectrum of riboflavin solutions in the presence of purines. Riboflavin, ●—●; riboflavin in 0·05 M-adenosine, ○—○; riboflavin in 0·052 M-caffeine, ×—×. Riboflavin concentration $1{\cdot}07 \times 10^{-5}$ M.

Effect of pH *on purine quenching.* The quenching efficiency of caffeine on riboflavin is not affected by a change in pH of the solution. Adenine or adenosine, on the other hand, lose much of their quenching ability in acid media. If the pH of a solution of riboflavin containing a fixed amount of adenosine (Fig. 4) is plotted against the intensity of the fluorescence, a maximum of the latter is attained at pH 2·6–2·8. This maximum is due to a combination of two simultaneous effects, the decrease in the quenching efficiency of adenine and the direct quenching of the fluorescence by the hydrogen ions. The last effect is discussed below.

Quenching of the fluorescence of riboflavin by acids and alkalis. The fluorescence of riboflavin vanishes in strongly acid or alkaline solutions, a fact that has been explained by the assumption that the fluorescent form is the dipolar ion while the cationic and anionic forms are completely non-fluorescent (Kuhn & Moruzzi, 1934). These authors calculated the dissociation constants of the two forms on the assumption that the degree of dissociation, $\alpha = I/I_0$, in every case. As we have seen, this is a fair approximation only if the lifetime of the excited state of the fluorescence is not appreciably changed on quenching.

Table 4. *Effect of the hydrogen-ion concentration on the intensity and polarization of the fluorescence of riboflavin*

(Solvent: glycerol-water mixture of viscosity 0·031 c.g.s. unit at 17°; E is the extinction value at 450 mμ. The last column of the Table is the degree of dissociation of the anionic form of riboflavin calculated on the assumption $\alpha = E/E_0$, E_0 being the value of the extinction in the absence of acid.)

HCl concn. (M)	I_0/I		τ_0/τ	$E_{450\,m\mu}$	$\alpha = E/E_0$
0	1	0·027	1	0·080	1
0·62	3·31	0·080	3·34	—	—
1·25	5·44	0·123	5·72	0·075	0·94
2·50	10·7	0·176	9·7	0·063	0·79
3·75	19·2	0·224	14·5	0·058	0·72
5·00	38·5	0·254	18·7	—	—
6·25	50	0·265	20	0·050	0·62

The quenching of the fluorescence of riboflavin by hydrochloric acid showed a shortening of the lifetime of the excited state much the same as that found in the quenching by potassium iodide. The results are given in Table 4. It is apparent that equation 4 cannot be applied to the quenching of riboflavin by

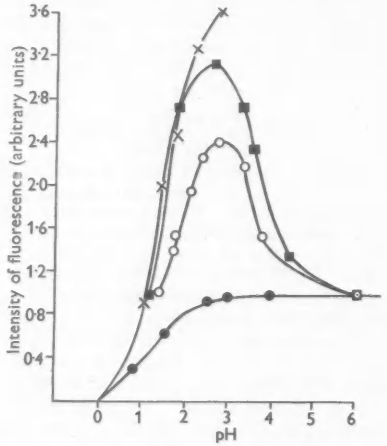

Fig. 4. Effect of pH on the fluorescent efficiency of FAD and of riboflavin in the presence of quenchers. ×—×, riboflavin; ■—■, FAD; ○—○, riboflavin in 0·05 M-adenosine; ●—●, riboflavin in 0·05 M-caffeine. Concentration of riboflavin of the solutions 5×10^{-6} M-concentration of FAD is the same. (Determined by the absorption at 454 mμ.)

hydrogen ions. It is not surprising, therefore, that Michaelis, Schubert & Smythe (1936), who used a spectrophotometric method, obtained for the pK of the anionic form of riboflavin a value of −0·2, while Kuhn & Moruzzi (1934), using the fluorimetric method, obtained pK = 2. Table 4 shows that, when five-eighths of the original riboflavin dipolar ions are present in the solution, the fluorescence efficiency has been reduced to one-twentieth of the original. This means that at this particular hydrogen-ion concentration, less than one-tenth of the excited molecules emit radiation, the other nine-tenths passing into the non-fluorescent form before emission can take place. The shortening of the mean life is thus explained. The molecules which emit are those which have such a short lifetime of the excited state that they manage to fluoresce before passing into the non-fluorescent species. In this case, therefore, there is little doubt that the value of Michaelis *et al.* (1936) is correct.

Table 5. *Effect of potassium hydroxide on the intensity and polarization of the fluorescence of riboflavin*

(Solvent: glycerol-water mixture of viscosity 0·0658 c.g.s. unit at 16°. The solutions were kept in the dark up to the time of the observations, to avoid the well known photochemical splitting of lumiflavin. The calculated polarizations were obtained by introducing the experimental value of I_0/I into equation 5, assuming $\Sigma/\tau_0 = 5$, solving for τ_0/τ and using this last value in equation 2 to obtain $1/p$.)

	Polarization	
I_0/I	Calculated	Observed
1	—	0·067
2·18	0·076	0·070
5·08	0·091	0·082
10·4	0·112	0·110
16·4	0·122	0·122
23·8	0·151	0·151

With potassium hydroxide it is found that the lifetime of the excited state changes slowly on quenching. From equation 5 it is found that the mean life of the cationic form is approximately 5×10^{-8} sec. Data for a solution of riboflavin quenched by potassium hydroxide are given in Table 5. Applying equation 5, half dissociation would occur, not at $I/I_0 = 0{\cdot}50$, but at $I/I_0 = 0{\cdot}42$; so that the error in the pK value of the cationic form obtained by Kuhn & Moruzzi (1934) is not large.

Quenching of the fluorescence of riboflavin by silver ions. When silver nitrate or silver acetate is added to a solution of riboflavin in distilled water the solution

immediately turns red and the tint becomes deeper as time passes showing that a slow reaction is taking place. At the same time the fluorescence drops accordingly. On heating the solution the colour changes, approaching that of a solution of free riboflavin, and the fluorescence reappears. On cooling, the solution again becomes red and less fluorescent, and this cycle of events can be repeated at will. Acidification destroys the colour and on neutralization with ammonia the red colour returns and persists at alkaline pH. Addition of a silver precipitant (chloride or phosphate ion) to the cold solution results in a brown precipitate, but addition of the same precipitant to the boiling solution produces only the usual precipitate of silver chloride or phosphate leaving as supernatant a solution of riboflavin with its full fluorescence.

Clearly we have here the formation of a complex which dissociates on boiling and reassociates on cooling. The formation of the complex takes place slowly and the same is true of its dissociation. The determination of the dissociation constant by the fluorimetric method is made difficult by the change in the absorption spectrum on addition of silver. The polarization of the fluorescence of riboflavin showed no increase on quenching by silver ions.

Fluorescence of flavin-adeninedinucleotide (FAD)

FAD was prepared by the method of Warburg & Christian (1938). The preparations were taken to the stage of extraction from the cresol solution. The absorption spectrum in the visible and near ultraviolet ($\lambda > 300$ mμ.) corresponded exactly to that of *iso*alloxazine. At least 95% of the absorption at 450 mμ. disappeared on addition of dithionite, and the amount of FAD determined in this way agreed well with the value obtained by the D-amino-acid oxidase method (Warburg & Christian, 1938). Paper chromatograms (Crammer, 1948) showed that FAD was the only flavin present. The fluorescence which varied slightly in the different preparations, due to impurities able to absorb the exciting light, was approximately 0·2 of the fluorescence of riboflavin solutions of the same molar concentration.

Effect of pH *on the fluorescence efficiency of FAD.* When the fluorescence intensity of the solutions is plotted against the pH a striking difference is observed between riboflavin and FAD. While the fluorescence of the former falls slowly between pH 6 and 4 and then rapidly, the solution of FAD at pH 4·5 already shows an increase in the fluorescence efficiency and at pH 3 this increase is strong enough to be detectable in daylight by the naked eye. The curve of Fig. 4 shows a well defined maximum at pH 2·7–2·8. Comparison with the pH intensity curve of a solution of riboflavin of the same molar concentration indicates that below pH 1·5 the fluorescence of both substances is the same. This effect is not due to a destruction of the dinucleotide, which is not to be expected at this acidity (Warburg & Christian, 1938). If, after standing at pH 2·8 for 24 hr., the solution is brought back to pH 5 the fluorescence drops exactly to the value corresponding to the latter pH, showing the complete reversibility of the phenomenon.

Effect of pH *on the absorption spectrum.* Comparison of the absorption spectrum of solutions of FAD at pH 7 and 2·7 (phosphate and acetate buffers respectively) showed in the latter a noticeable increase in the absorption maximum at 450 mμ., but no change either in the 375 mμ. maximum or in the position of the red edge of the absorption band.

Mean life of the excited state of the fluorescence of FAD. The purpose of the following experiment was to ascertain whether the diminished fluorescence of FAD, as compared with that of riboflavin, was accompanied by a corresponding shortening of the lifetime of the excited state. The latter was measured by the method of Perrin (1926) which is based on the polarization of the fluorescence in media of different viscosities. According to Perrin (1926)

$$\frac{1}{p} = \frac{1}{p_o} + \left(\frac{1}{p_o} - \frac{1}{3}\right) \frac{RT}{\eta V} \tau_0,$$

where p is the polarization of the fluorescence of a substance of mean life in the excited state τ_0, and

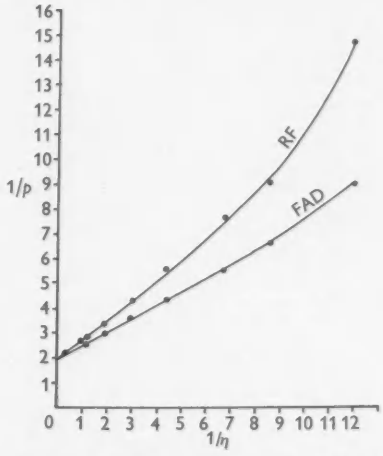

Fig. 5. Polarization of the fluorescence of riboflavin (RF) and FAD in glycerol water mixtures of increasing viscosity. $1/p$, reciprocal of the polarization; $1/\eta$, reciprocal of the coefficient of viscosity in c.g.s. units. Concentration of the fluorescent substances, approximately 2×10^{-6} M.

molar volume V, in a medium of viscosity η, kept at the absolute temperature T; R is the gas constant and p_o the limiting, or fundamental, polarization. If

$1/p$ is plotted against $1/\eta$ a straight line is obtained which, extrapolated to $1/\eta = 0$, cuts the ordinate at $1/p = 1/p_o$.

In Fig. 5 the polarization of the fluorescence of solutions of riboflavin (RF) and FAD in glycerol-water mixtures of different viscosities is shown, $1/p$ being plotted against $1/\eta$. Both substances yield $1/p_o \simeq 2$. Both give straight lines at high viscosities, and at low viscosities both deviate from the simple Perrin law. This occurs with practically all the fluorescent substances of low molecular weight studied (Perrin, 1926; Wawilov, 1936). Over the linear portion of the graph where the Perrin (1926) equation is followed, it is easy to see that the ratio of the slopes of the riboflavin and FAD curves is

$$\frac{S_{RF}}{S_{FAD}} = \frac{V_{FAD}\,\tau_{RF}}{V_{RF}\,\tau_{FAD}} = \frac{11}{8}.$$

This ratio approximates to that of the molecular volumes, and consequently

$$\tau_{RF} \simeq \tau_{FAD}.$$

If we take for τ_{RF} the value of 1×10^{-8} sec., obtained by the method of Wawilov (1928), we get for the molecular volume of riboflavin in solution $V_{RF} = 550$ ml. which seems a reasonable value, to judge by the constitution, and the molecular volume of substances of similar configuration (Marinesco, 1927 *a*, *b*).

Effect of pH *on the polarization of the fluorescence of FAD.* Changing the pH from 6 to 2·7 did not change the polarization of the fluorescence of FAD in a glycerol-water mixture, although the brightness of the fluorescence increased by about 300 %.

DISCUSSION

Examination of the mode of action of the purine quenchers leaves no doubt that the formation of a complex between flavin and purine takes place, and that the complex has a heat of dissociation of 1–2 kg.cal./mol., a mean life no shorter than 10^{-6} sec., and an absorption spectrum differing appreciably from that of free flavin. The differences between caffeine on one side, and adenosine or adenine on the other in the effect of pH on the quenching, can be attributed to the presence of the amino group in the last two named substances. According to Levine & Simms (1926), the pK of this group is about 3·7, corresponding to the region of rapid increase in the fluorescence efficiency of FAD shown in Fig. 4.

The quenching effect of purines on the fluorescence of certain hydrocarbons has been shown by Weil-Malherbe (1946), who was able to isolate some of the more stable of these complexes. The evidence presented in this paper is based on the thermal coefficient of the quenching, the effect on the life of the excited state and the changes in the absorption spectrum. The effect on the mean life of the excited state is decisive in differentiating the quenching by complex formation from that due to collisions of the second kind, although there is a smooth transition between these two types (Weber, 1948). The reaction of riboflavin with silver ions represents a limiting case between the formation of a non-fluorescent complex and a non-fluorescent stable substance.

Quenching by hydrogen chloride, on the other hand, shows that when a fluorescent form in solution is in equilibrium with its non-fluorescent conjugate, the apparent fluorescent intensity bears no direct relation to the amount of fluorescent form present. Thus the value obtained by Kuhn & Moruzzi (1934) for the pK of the anionic form of riboflavin can be shown to be considerably in error. Whenever the simple equation 4 is applied to an equilibrium of this type, it is necessary to ascertain previously that the lifetime of the excited state is not changed by addition of quencher. The fluorescence of FAD and its variation with pH are readily correlated with similar changes taking place in riboflavin solutions in the presence of adenine or adenosine. There is no doubt of the existence of an internal complex in FAD, between the alloxazine and the purine parts of the molecule. The presence of some residual fluorescence in FAD may seem puzzling. The simplest explanation is that the non-fluorescent form is in equilibrium with a fluorescent one, and that this equilibrium is displaced by a change of pH. Since the lifetime of the excited state is the same in riboflavin and FAD, it must be concluded that the non-fluorescent internal complex has a long mean life, as found for the adenosine-riboflavin complex. The argument in favour of this internal complex is confirmed by the resemblance between the absorption spectrum of FAD and that of riboflavin in the presence of purines.

When FAD or riboflavin phosphate combines with an enzyme protein, in some cases (notatin) complete disappearance of the fluorescence has been noted (Keilin & Hartree, 1946), while in other cases (diaphorase) the fluorescence remains unchanged (Straub, 1939). Proteins, as such, display no conspicuous quenching properties, so that the disappearance of the fluorescence must be sought in the place and mode of attachment of the prosthetic group. Aromatic groups in the protein (tyrosine) are the more likely to act as quenchers. However another explanation is possible when FAD is the prosthetic group; union with the protein might simply prevent the complex from reverting to the fluorescent form in notatin, while for diaphorase the ease of this transition would not be affected.

Adenine and adenosine, and purines in general, are able to deactivate with quenching many fluorescent molecules. It is not perhaps unreasonable to

assume that other forms of electronic energy, not connected with the radiative transition but with other degrees of freedom, might easily be absorbed by purines. This would lead to the existence, in certain degrees of freedom of the purine molecule, of an energy content in excess of that which could be accounted for by a random distribution and the principle of the equipartition of energy. The presence of adenine in several prosthetic groups and coenzymes, where the purine seems to play no part in the catalysed reaction, is an indication that some general property of this type of compound, such as the one suggested, may be involved.

SUMMARY

1. A study of the fluorescence of riboflavin and of flavin-adeninedinucleotide, and the effect of different quenchers on the former has been made.

2. The quenching of the fluorescence of riboflavin by purines is due to the formation of a complex having a heat of dissociation of $1{\cdot}6 \pm 0{\cdot}6$ kg.cal./mol. and a lifetime longer than 10^{-6} sec.

3. The small fluorescent efficiency of flavin-adeninedinucleotide in comparison with that of riboflavin is due to the formation of an internal complex between the *iso*alloxazine and the adenine of the molecule. The complex is non-fluorescent and exists in equilibrium with its fluorescent form, the equilibrium being easily displaced by changes in pH.

4. A study of the quenching of the fluorescence of riboflavin by acids and alkalis, shows that the fluorimetric method cannot be used for the determination of the dissociation constant of the anionic form of riboflavin, but its use is justified in the case of the cationic form.

5. A complex of riboflavin with silver ions is described.

6. The variations in the fluorescence of flavoproteins is discussed, and a relation between the quenching properties of adenine and its presence in prosthetic groups and in coenzymes is suggested.

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The Cozymase of Mammalian Brain

By M. GORE, F. IBBOTT and H. McILWAIN
Biochemical Laboratories, Institute of Psychiatry (British Post-graduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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The cozymase of brain has been studied because of the central importance of this compound in carbohydrate metabolism, and also because in brain as a whole cozymase exists together with very active systems which degrade it. Inhibition of the degradation appears to be accompanied by disturbance of the balance of carbohydrate metabolism (McIlwain & Grinyer, 1950). The present paper describes studies of the normal cozymase content of mammalian brain, of the stability of cozymase in the tissue during various manipulations, and of its possible origin.

EXPERIMENTAL

Determination of cozymase

Apozymase system

Preparation of apozymase from yeast, and its use for determining cozymase, were based on the descriptions of Myrbäck (1933), Euler (1936), Axelrod & Elvehjem (1939) and McIlwain & Hughes (1948).

Apozymase. Fresh bottom yeast obtained from Barclay Perkins and Co. Ltd., to whom we are indebted, was sucked as dry as possible on a Büchner filter, pressed through a wire sieve (0·1 in. mesh) and dried overnight by a fan. The

dried yeast was stored over $CaCl_2$ at 4°; its activity declined after 2–3 months' storage. For the preparation of apozymase, it was washed and dried as described by Axelrod & Elvehjem (1939). With some batches of dried yeast, two washings between each drying were sufficient to remove the cozymase and to yield preparations with high fermentation rates when cozymase was added, but with low rates without cozymase. The apozymase was prepared weekly and stored in a refrigerator over $CaCl_2$. The rate of fermentation of glucose by apozymase preparations declined after about 10 days' storage.

Hexose diphosphate. Hexose diphosphate was obtained commercially as the Ba salt, and one batch was prepared by the method of Neuberg & Lustig (1942). Commercial samples were fractionally precipitated by ethanol at pH 4·0 (Robison & Morgan, 1930), to reduce their content of inorganic phosphate. A solution of Na hexose diphosphate was prepared each day. The Ba salt was dissolved in ice-cold N-HCl, 2 M-Na_2SO_4 added in slight excess to remove the Ba, the solution brought to pH 6·3 with N-NaOH, diluted with water so that the organic P was 10 mg./ml., and centrifuged.

Cozymase estimation was carried out at 30° with Warburg manometers and conical single side-armed flasks. The main compartment contained hexose diphosphate, glucose and Mg and Mn salts in the concentrations recommended by Axelrod & Elvehjem (1939). The final phosphate concentration of the reaction mixture was 0·065 M, which was the optimum concentration for the strain of yeast used. The solution whose cozymase was to be determined was placed in the side arm and the final volume brought to 2 ml. with water. Apozymase (0·2 g.) was added in powder form to each flask immediately before incubation commenced.

After equilibration at 30° for 9 min. the flasks were tipped and readings taken at 3 min. intervals for 21 min. and then at 5 min. intervals. After a short induction period, the CO_2 evolution in each vessel reached a steady rate which was maintained for 20–25 min. The rate of evolution of CO_2 was proportional to the amount of cozymase added to the reaction mixture up to quantities of 15 or 20 μg. pure cozymase, but it was found convenient to make most of the estimations in the range 1–5 μg. cozymase.

A control flask without added cozymase and one containing standard cozymase were included in each set of estimations. A specimen of cozymase obtained commercially and found to be 53% pure was usually employed as standard, and results were calculated in terms of pure cozymase. The cozymase content of the standard specimen was determined as described by McIlwain & Rodnight (1949) and was found to agree approximately with the value obtained with Slater's (1950) method, for details of which we are indebted to Mr C. Kratzing and Dr E. C. Slater. The activity of the enzyme system (rate of evolution of CO_2/μmol. cozymase) varied with different apozymase preparations, which were therefore not suitable (as implied by McIlwain & Hughes, 1948) for assessing the purity of a cozymase specimen without the use of a standard specimen of known purity. Values ranged from 3 to 8 μmol. CO_2/μg. cozymase/hr. with an average of 5 μmol. CO_2/μg. cozymase/hr. The system was found to estimate dihydrocozymase as cozymase. Adenosinetriphosphate, nicotinamide and nicotinic acid did not affect the determinations.

Extraction of cozymase

The cozymase content of tissue extracts and fresh tissues has been studied in the present investigation. Phosphate buffer (0·1 M, pH 6·2) has been used as the basis of the extracting fluid because it formed part of the apozymase system which was used for the estimations of cozymase, and also because pH 6·2 is close to the pH of optimum stability of cozymase. Heating in this buffer alone for 15 min. at 70° was found to cause little destruction of cozymase. Good recoveries of cozymase added to saline extracts of fresh brain homogenates, or to acetone-dried brain, were obtained after this treatment (Table 1).

This procedure, however, was found to be quite inadequate for the extraction of cozymase from fresh tissue. It was suspected that the loss might be due in part to the action of the enzyme degrading cozymase; nicotinamide which inhibits the action of this enzyme was therefore added to the buffer solution used for extraction. Nevertheless, only 6% of the cozymase added to a brain homogenate was recovered, after heating for 15 min. at 70° in phosphate buffer at pH 6·2 which contained 0·1 M-nicotinamide (Table 1). It was found, however, that by heating for only 2 min. at 100° 90–94% recovery of added cozymase could be obtained. The presence of the nicotinamide was important: it considerably improved the yield of cozymase from fresh tissue (Table 2). For extraction of fresh tissue and tissue slices, a small piece of weighed tissue was dropped into the extracting fluid already at 100°, broken with a glass rod, and kept at 100° for 2 min. Tissue homogenates were pipetted directly into the extracting fluid at 100°.

The efficiency of the methods of extraction has been judged also by comparing the yields of cozymase from fresh tissue, with their content of nicotinic acid after hydrolysis (see below). In computing cozymase content, no correction has been made for the possible loss of some 8% during extraction.

Table 1. *Recovery of cozymase added to brain preparations*

(The extracting fluids consisted of a 0·1 M solution of nicotinamide in phosphate buffer, pH 6·2, of a molarity which gave a final phosphate concentration of 0·1 M. To this buffer, in small tubes in a bath at the temperature of extraction, were added the tissue preparations with or without additional cozymase.)

		Cozymase			
Preparation	Extraction	In tissue (μg.)	Added (μg.)	Recovered (μg.)	Recovery (%)
Saline extract of fresh tissue	15 min. at 70°	0	2·35	2·30	98
Saline extract of acetone-dried tissue	15 min. at 70°	0	3·75	3·75	100
Tissue homogenate	15 min. at 70°	0	14·7	0·9	6
Tissue homogenate	2 min. at 100°	1·93	3·0	4·76	94
Tissue slice	2 min. at 100°	2·0	2·5	4·35	93

Determination of nicotinic acid

Determinations of the total nicotinic acid content of guinea pig cerebral cortex were made on homogenates after hydrolysis of the tissue for 1 hr. at 100° in 2 N-HCl. The supernatant solution after hydrolysis was decolorized by zinc hydroxide precipitation according to Friedemann & Barborka (1941) and nicotinic acid determined as described by McIlwain & Rodnight (1949).

RESULTS

Cozymase content of different tissue preparations

Cozymase and nicotinic acid. The cozymase content of different preparations of guinea pig brain is given in Table 2. Values for fresh brain were obtained by killing the animal by a blow on the neck, bleeding and removing the brain immediately. A portion of cerebral hemisphere, approx. 0·1 g., was weighed as rapidly as possible on a torsion balance, dropped into phosphate buffer pH 6·2 containing nicotinamide at 100°, broken up, and the whole cooled after 2 min. The cozymase content of the guinea pig cerebral hemisphere averaged 159 μg./g. wet weight. Lower values for cozymase were obtained under other conditions. Thus, when the concentration of nicotinamide in the extracting fluid was 0·04 M, the yield of cozymase was only 100 μg./g. Extraction in water for 2 min. at 100° yielded only 8 μg. cozymase/g. fresh weight.

Table 2. *Cozymase content of brain preparations*

(Extraction conditions, except where otherwise indicated, as described in Table 1, heating 2 min. at 100°.)

		Cozymase found			
Brain preparation	Extraction	No. of samples	Mean (μg./g. fresh tissue)	Standard deviation (μg./g. fresh tissue)	Mean (μmol./g. fresh tissue)
Guinea pig cerebral hemisphere	0·1 M-phosphate with nicotinamide 0·1 M	7	159	11·7	0·240
Rat cerebral hemisphere	0·1 M-phosphate with nicotinamide 0·1 M	12	158	16·3	0·240
Mouse cerebral hemisphere	0·1 M-phosphate with nicotinamide 0·1 M	4	333	25·8	0·505
Rabbit cerebral hemisphere	0·1 M-phosphate with nicotinamide 0·1 M	—	103	—	0·153
Guinea pig cortex slices, fresh	0·1 M-phosphate with nicotinamide 0·1 M	7	152	16·3	0·230
Guinea pig cortex slices, suspended in Krebs-Ringer solution for 5 min.	0·1 M-phosphate with nicotinamide 0·1 M	20	109	16·4	0·165
Guinea pig cortex slices, fresh	0·1 M-phosphate	4	45	4·6	0·068

Tissue metabolism

The methods described by McIlwain & Grinyer (1950) were followed for cutting and handling tissue slices, preparation of saline solutions, determination of phenosafranine, measurement of respiration and lactic acid formation, and manometric determination of the course of acid formation. When cozymase was determined in slices after incubation in fluids without added cozymase or dihydrocozymase, the slices (usually 0·35 mm. thick and of 50–60 mg. moist weight) were picked from the fluids with a mounted, bent platinum wire, drained against the side of the vessel and dropped into 1 ml. of the heated extracting solution. The cozymase content of the fluid used in incubation, under these conditions, has been found to be extremely small, although that of the tissue may be well maintained. For determining the cozymase of slices which had been incubated with cozymase and dihydrocozymase, the same procedure was followed if the initial cozymase level of the solution was not much greater than that expected in the slice. When the initial cozymase or dihydrocozymase concentrations were much higher than those in the slice, the slice was transferred to about 10 ml. of 0·9% NaCl for 1–2 sec. (ensuring that it opened in the saline) and was then drained and dropped into the extracting fluid. Homogenates were made in test-tube homogenizers from the whole cerebral hemispheres, with three times their volume of suspending fluid. Weights of tissue, when not otherwise specified, refer to fresh weights or those immediately after dissection. When slices of tissue were weighed after cutting or placing in saline, and then draining from the saline, the result is referred to as a moist or wet weight.

Table 3. *Content of cozymase and of total nicotinic acid in brain*

Preparation	Nicotinic acid (μmol./g. fresh wt.)	Cozymase (μmol./g. fresh wt.)
Rabbit cerebral hemisphere	0·204	0·153
Guinea pig cerebral hemisphere	0·275	0·257
Rat whole brain	0·260	0·240
Mouse whole brain	0·590	0·505

It has been necessary to consider carefully the adequacy of this method of preparing the tissue for analysis, in view of the rapid degradation of cozymase in damaged tissue. For this purpose, the levels of cozymase have been compared with those of the total nicotinic acid of the same tissue preparations

(Table 3). The values showed that cozymase, as extracted by the present method, accounted for 93% of the total nicotinic acid in guinea pig brain. Corresponding values were: rat 92, mouse 85, and rabbit 75%. Breakdown of the nicotinamide nucleus during these experiments is considered unlikely, and at least in the case of guinea pig and rat, therefore, the method of preparation of the specimen does not lead to significant loss of cozymase.

Cozymase in different animals. Species differences in the cozymase content of brain tissue have been observed by Axelrod, Elvehjem & Spies (1941), and in the total nicotinic acid content of brain after enzymic hydrolysis by Taylor, Pollack & Williams (1942). The cozymase content of guinea pig cerebral hemispheres found by us (159 μg./g.) is greater than the value given by Axelrod *et al.* (1941) (107 μg./g.), while the value found by us for rat-brain cozymase content (156 μg./g.) was less than half the value given by Axelrod *et al.* (1941) or that corresponding to the total nicotinic acid level in rat brain given by Taylor *et al.* (1942). Taylor *et al.* used a Wistar strain of rats; those used by us were Norwegian 'hooded' rats. This difference in strain may account for the difference in cozymase level. The total nicotinic acid content of mouse brain recorded by Taylor *et al.* (1942) corresponds closely to our value for cozymase in mouse brain (333 μg./g.). It is observed that with the four species which we have examined the cozymase content of the brain tissue seems to decrease with increase in brain size.

Cozymase in slices and homogenates. Cozymase was found to be relatively stable in guinea pig cortex slices. When slices approximately 0·35 mm. thick and 50 mg. in weight were extracted immediately on section, their cozymase content ranged from 135 to 176 μg./g. wet weight with an average of 152 μg./g. wet weight. Tissue slices which had been placed, as they were cut, into an aerated bath of Krebs-Ringer phosphate-glucose medium for 5 min. prior to use in an experiment had cozymase contents of 82–132 μg./g. wet weight (average 109 μg./g.).

Homogenates prepared from guinea pig cerebral hemispheres in 0·1 M-nicotinamide gave cozymase contents corresponding to 127 μg./g. fresh weight (Table 4). This level fell to 86 μg./g. if the homogenate was kept at 20° for 15 min. In the absence of nicotinamide the cozymase level in a tissue homogenate dropped, by the time it could be pipetted into extracting fluid, to approximately 25% of the cozymase present in fresh tissue. Following this, the cozymase content of the homogenate remained relatively constant for at least 30 min. The nature of the fluid in which the homogenate was prepared affected the level to which the cozymase concentration fell. It appeared that the residual cozymase might be bound in some subcellular structure, protected from attack by the cozymase-splitting enzyme of brain but liberated by the extraction.

Table 4. *Cozymase in brain homogenate*

(Guinea pig cerebral hemispheres were homogenized in 3 vol. liquid, and then extracted at the times stated in nicotinamide (0·1 M final conc.) in 0·1 M-phosphate buffer pH 6·2 at 100°.)

Homogenate kept at 20° prior to extraction for (min.)	Cozymase content (μg./g. fresh wt.) after homogenizing in			
	0·1 M-Nicotinamide	Physiological saline (Krebs & Henseleit, 1932)	0·9% NaCl	Water
0	127	—	36, 52	—
5	115	—	46, 52	—
10	—	—	46, 49	—
15	86	—	52	—
20	—	—	42	—
30	—	45, 70	30, 41	10, 30

Cozymase content of slices during in vitro *metabolism*

The preceding experiments suggested that slices could be cut from brain cortex with little loss of their cozymase. Subsequent to this, however, even leaving in saline for short periods caused some loss. To study the maintenance of cozymase during typical metabolic procedures, slices were placed, as cut, in aerated saline solutions containing various substrates. After cutting sufficient slices for an experiment, one after another of them was drained from the saline and weighed on a torsion balance. Some were transferred immediately to the nicotinamide-phosphate at 100°, and others put into saline solutions of different substrates, in the main compartments of Warburg vessels. Gas exchange was followed at 37°, and after different intervals slices were removed from the vessels with a mounted bent platinum wire and transferred to the extracting fluid at 100°.

Cozymase was found to be relatively stable during the first 70–90 min. under a variety of conditions (Table 5). Aerobically, with glucose in phosphate saline its level fell to about two-thirds of the initial

value in the first 80 min. The fall was not significantly greater if the substrate was omitted; the slice continued to respire, in the absence of substrate, at about half the rate of its respiration in the presence of glucose. Pyruvate and glutamate maintained similar levels of respiration and of cozymase. In several experiments the cozymase of slices was compared after incubation (*a*) anaerobically and without added glucose, and (*b*) aerobically with glucose. Cozymase levels were found to be lower in the absence of glucose and oxygen.

When slices, whose cozymase content had been lowered in this way, were further incubated aerobically and with glucose or glutamate, their cozymase continued to fall (Table 5, Exp. 4); their rate of respiration was also low. Slices pretreated in this way and then incubated anaerobically with glucose also continued to lose cozymase; their rate of glycolysis was less than 5% of that of fresh slices and their cozymase content about 40%.

decrease in the cozymase of the slices. Effects remained small in phosphate and bicarbonate buffers during aerobic reaction with glucose, glutamate, pyruvate, and a mixture of pyruvate and fumarate. Some anaerobic reactions were also studied (Table 5). The change produced by phenosafranine was, however, consistently a fall in cozymase level. During the present experiments, phenosafranine was absorbed by the slices as has been described by McIlwain & Grinyer (1950).

Addition of cozymase and dihydrocozymase to slices. In considering how the normal cozymase content of brain tissues might be maintained, assimilation of cozymase was examined (Table 6). When slices as ordinarily prepared for metabolic studies were incubated under various conditions, the cozymase level of those incubated in the presence of added cozymase differed little if at all from those incubated in its absence. Moreover, if slices were first depleted in cozymase by anaerobic incubation, and cozymase

Table 5. *Cozymase of guinea pig brain cortex slices after metabolism in presence and absence of phenosafranine*

(Results similar to those of Exp. 2 were obtained in the absence of substrate and results similar to those of Exp. 3 were obtained with pyruvate (0·017 M) and with pyruvate and fumarate as substrates. Respiration during the aerobic experiments was of the order of 70–95 μmol./g. moist wt./hr. and was little (less than 10%) affected by phenosafranine. Phenosafranine, however, greatly increased aerobic acid formation from glucose.)

Exp. no.	Metabolic conditions (O_2, aerobic; N_2, anaerobic)	Period of metabolism (min.)	Cozymase content of slices (μg./g. moist wt.) after metabolism in: Medium alone	Cozymase content of slices (μg./g. moist wt.) after metabolism in: Medium with 10^{-5} M-phenosafranine
1	Phosphate; glucose; O_2	0	171	—
		40	128	134
		80	114	87
2	Bicarbonate; glucose; O_2	0	120	—
		90	79	57
	Bicarbonate; glucose; N_2	90	61	60
3	Bicarbonate; glutamate; O_2	0	120	—
		75	85	74
	Phosphate (no substrate); O_2	0	120	—
		75	82	—
	Phosphate; glutamate; O_2	0	120	—
		75	89	77
4	After initial anaerobic incubation in phosphate, no substrate, anaerobically	0	64	—
		60	26	—
	As above, with glutamate	30	30	21
		60	26	36

Phenosafranine. Although this substance (see below, p. 127) causes large changes in the aerobic carbohydrate metabolism of brain-cortex slices, it did not have a large effect on their cozymase levels. In Table 5, Exp. 2 illustrates this: a concentration of phenosafranine which increased aerobic lactic acid formation three- to six-fold, caused a small

added later, aerobically or anaerobically, little additional cozymase could be detected in the slice (Exp. 3, Table 6).

Assimilation of dihydrocozymase was also examined as it is stable in the presence of the degrading enzyme of brain and also it is less polar than cozymase. Results, however, were similar (Table 6). In

Table 6. *Cozymase content of slices after incubation in the presence of cozymase and dihydrocozymase*

(Slices (weighed) of 50–70 mg. were incubated at 37° in 3 ml. of saline containing the stated weights of coenzyme. When two periods of incubation were arranged, under different conditions, these followed within 1–5 min. of each other. Results similar to those of Exp. 1 were obtained after 180 min. incubation, and similar to those of Exp. 2 after preliminary incubation anaerobically in phosphate-glucose, and with the addition of either Co I or Co I H_2.)

Exp. no.	Conditions of incubation		Cozymase of slice (μg./g. moist wt.)		Duration of second period (min.)
	First period	Second period	At beginning of second period	At end of second period	
1	None	Phosphate-glucose; air	120	67	90
		Phosphate-glucose; Co I H_2, 670 μg.	120	82	90
2	Bicarbonate-glucose; N_2; 140 min.	Bicarbonate-glucose; N_2	48	37	60
		Co I H_2, 27 μg.	48	40	60
		Co I H_2, 134 μg.	48	48	60
3	Phosphate-glucose; N_2; 120 min.	Phosphate-glucose; air	42	42	20
			42	35	60
		Phosphate-glucose; Co I H_2, 100 μg.	42	41	20
			42	34	60

experiments (2) and (3) of Table 6, most of the dihydrocozymase added was present in the fluid in which the slices had been incubated. It had, therefore, not been oxidized and degraded, or otherwise changed, but nevertheless it was not assimilated into the slices.

Attempts to increase cozymase content of slices by adding possible precursors. Attempts have been made to obtain a synthesis of cozymase in brain slices by incubating them in a medium containing mixtures of various constituents of the cozymase molecule. Kohn (1938) and Axelrod *et al.* (1941) had observed an increase in the cozymase content of red blood cells of pellagrins while they were being treated with nicotinic acid, and Kohn & Klein (1939, 1940) obtained a synthesis of cozymase in red blood cells *in vitro* by incubating them in a medium containing nicotinic acid. The acid, in this respect, was superior to the amide. For this reason nicotinic acid, as well as the amide, has been included in our reagents. Guinea pig brain slices were incubated aerobically at 37° for 30 or 120 min. in physiological salines buffered at pH 7·4 with 0·02 M-phosphate, containing 0·01 M-glucose, with and without the addition of 0·067 M-nicotinamide, and 0·0033 M-nicotinic acid, ribose and adenosinetriphosphate. No consistent evidence of synthesis was obtained when cozymase was determined by the apozymase system.

In other experiments, so that a small synthesis of cozymase might be more easily detected, the tissue slices were incubated for 2·5 hr. anaerobically in the saline containing 0·01 M-glucose to reduce their original cozymase content. The average cozymase content of the tissue slices after this treatment was 45 μg./g. wet weight. After this anaerobic incubation of guinea pig slices, nicotinamide, nicotinic acid, ribose and adenosinetriphosphate or adenylic acid were added to the medium in final concentrations of 0·0033 M and aerobic incubation was allowed to proceed for 40 min. There was no detectable synthesis of cozymase in the tissue slices under these conditions nor when the experiment was repeated with 0·1 M-nicotinamide. This experiment was also carried out anaerobically in the presence of 0·01 M-glutathione with negative results.

Sodium glutamate (0·01 M) was used in place of glucose in two experiments. In addition to glutamate, the medium contained 0·1 M-nicotinamide and 0·0033 M-adenylic acid. The experiment was made both with fresh tissue slices and those in which the cozymase had been allowed to fall anaerobically, but no synthesis of cozymase was detected.

DISCUSSION

The present observations have partly answered the questions posed by the coexistence in brain of cozymase and a system which rapidly degrades it. The brain of guinea pigs or rats could be removed from the animal at room temperature and sampled (operations lasting 2–5 min.) without loss of more than 10 % of its cozymase. This was judged from the finding that the content of cozymase was about 92 % of the content of nicotinic acid. The maximal loss is thus some 10 %; some nicotinamide may normally exist in the tissue as such and some certainly exists as coenzyme II. On the other hand, in homogenates made at 0° the cozymase content fell to 20 % of the initial content of the tissue, within 2 min. The stability of cozymase in slices of the sort employed in studies of tissue metabolism was very much greater: some 60–70 % remained after slicing and floating in physiological salines, operations which occupied 5–10 min. On incubating aerobically under normal

manometric conditions an hour or so was needed for the level of cozymase in the slices to fall to 50% of that in the fresh whole brain.

Anaerobically, the cozymase of slices was depleted more rapidly, and conditions have not yet been found for its restoration. No evidence was found that cozymase was assimilated into either normal or depleted slices, or that dihydrocozymase, which was stable when added to slices and which might be considered more likely to penetrate them, was assimilated. Various mixtures containing nicotinamide, nicotinic acid, and adenine derivatives did not lead to increased cozymase. Examination of the possible route of synthesis is being continued. Differential determination of the oxidized and reduced forms of cozymase has not been attempted in the present study; the two were determined together by the apozymase system.

It was interesting to determine whether phenosafranine, which both increased the aerobic formation of lactic acid by brain-cortex slices (Dickens, 1936) and inhibited the degradation of cozymase in brain homogenates (McIlwain, 1949), affected the cozymase level of brain slices. The results showed that phenosafranine induced a slightly greater fall in the cozymase content of slices than that occurring in its absence. If the correlation suggested by McIlwain & Grinyer (1950) between the action of agents on cozymase degradation and on aerobic glycolysis reflects a real linkage between the two processes, then it is necessary to consider that the system, which in homogenates degrades cozymase, is functioning in slices without leading to loss of cozymase. This, and other observations of this and the preceding paragraph which are not yet understood, may be explained in terms of the complexity of the degradation and of other reactions undergone by cozymase.

SUMMARY

1. A method has been developed for determining the cozymase content of brain tissue.

2. The cozymase content of the cerebral hemispheres of mouse, rat, guinea pig and rabbit brain were determined. The values obtained were: mouse, 333, rat 158, guinea pig 159, rabbit 103 μg./g. fresh tissue respectively. The cozymase contained in guinea pig brain represents at least 92% of the total nicotinic acid in the tissue.

3. Cozymase was found to be relatively stable in brain slices. There was an average fall of 28% of the initial cozymase content of the tissue 10 min. after section and a much slower fall during active metabolism. The destruction of cozymase in homogenized tissue was very rapid. Only 25% of the initial cozymase remained 2 min. after homogenizing brain tissue in saline. The residual 25% cozymase seemed to be protected from the action of the enzyme degrading cozymase.

4. The increased glycolysis in brain-tissue slices produced by phenosafranine was not due to the maintenance of a high level of cozymase in the slices. In the presence of phenosafranine the fall in cozymase was rather greater than in its absence.

5. Attempts to synthesize cozymase in brain tissue slices by incubation with various components of the cozymase molecule did not lead to an increase in their coenzyme content, nor did incubation with cozymase and dihydrocozymase.

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